Effect of Dried Distillers Grains with Solubles (DDGS) on Cholesterol Metabolism and Xanthophylls Bioavailability in Rats

A Thesis
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Xuejiao Zhang

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Advisor: Daniel D. Gallaher, Ph.D.

Nov 2014

Acknowledgements

I would like to thank my advisor Dr. Daniel Gallaher for his guidance and support throughout my Master's program. His positive attitude encouraged me to overcome setbacks and finish my study. I learned a lot from him, not only academic knowledge but also living and working principles. I also want to thank my committee members, Dr. Jerry Shurson and Dr. Sabrina Trudo, for their support and advice.

I am grateful to staff helping me with LC-MS and GC, including Dr. Catrin Tyl, Dr. Jae Kyeom Kim, Dr. Jean-Paul Schirlé-Keller and Thomas Krick. Also, I greatly appreciate my lab mates: Cindy Gallaher, Drew Brockman, Ana Dos Santos, Sangyub Kim.

In the end, I want to express my appreciation to my families and friends. All my works cannot be achieved without your support and encouragement.

Dedication

I dedicate this dissertation to

my father, Zhe Zhang

and my mother, Zhimin Yang

for their love and support.

Abstract

Dried distillers grain with solubles (DDGS), a co-product generated from the fuel ethanol industry, contain the same nutrients found in corn, except for starch, which has been fermented to ethanol and carbon dioxide. The U.S. Fuel ethanol industry has experienced a rapid increase in production in the past decades with 5 million metric tons produced in 2000 to 42.5 million metric tons produced in 2013 [1]. Currently, DDGS is primarily used as a feed ingredient for livestock and poultry [2], and it has been recognized as an excellent source of energy, amino acids, water-soluble vitamins and minerals for poultry, swine [3, 4].

Considering its high concentration of nutrients, it may have a great potential value as an ingredient in human foods. Therefore we examined several potential human health benefits of DDGS. Specifically, the effect of feeding DDGS, as well as a corn bran fraction and DDGS soluble fraction, on liver cholesterol, total fat, oxidative stress and xanthophyll bioavailability in rats was examined. The results showed that diets containing DDGS and DDGS co-products decreased liver cholesterol, increased bile acids and total neutral sterol excretion, and promoted intestinal fermentation. However, there was no evidence that these products decreased oxidative stress. Due to lack of sensitivity in the methods used, we were unable to assess xanthophylls bioavailability.

Table of Contents

List of Tables

List of Figures

Chapter 1: Literature Review

Dried distillers grains with solubles (DDGS)

Description

Dried distillers grains with solubles (DDGS) is a co-product from fuel-ethanol industry. The official AAFCO （Association of American Feed Control Officials） definition for DDGS is: “Distillers Dried Grains with Solubles is the product obtained after the removal of ethyl alcohol by distillation from yeast fermentation of a grain or a grain mixture by condensing and drying at least ¾ of the solids of the resultant whole stillage by methods employed in the grain distilling industry” [5]. DDG is the dried grain residue after fermentation, which contains primarily protein, lipid, unfermented fiber, and minerals. Dried distillers grains with solubles (DDGS) is the mixture of DDG with the concentrated thin stillage to 10-12 percent moisture. Corn is the most commonly used grain in ethanol production, however, some wheat and sorghum are also used [2]. The increase in the demand for ethanol as a fuel additive has resulted in a dramatic increase in the amount of the DDGS and corn co-products produced. The production of corn co-products from dry-grind ethanol production was 34.4 million metric tons in 2013 [2].

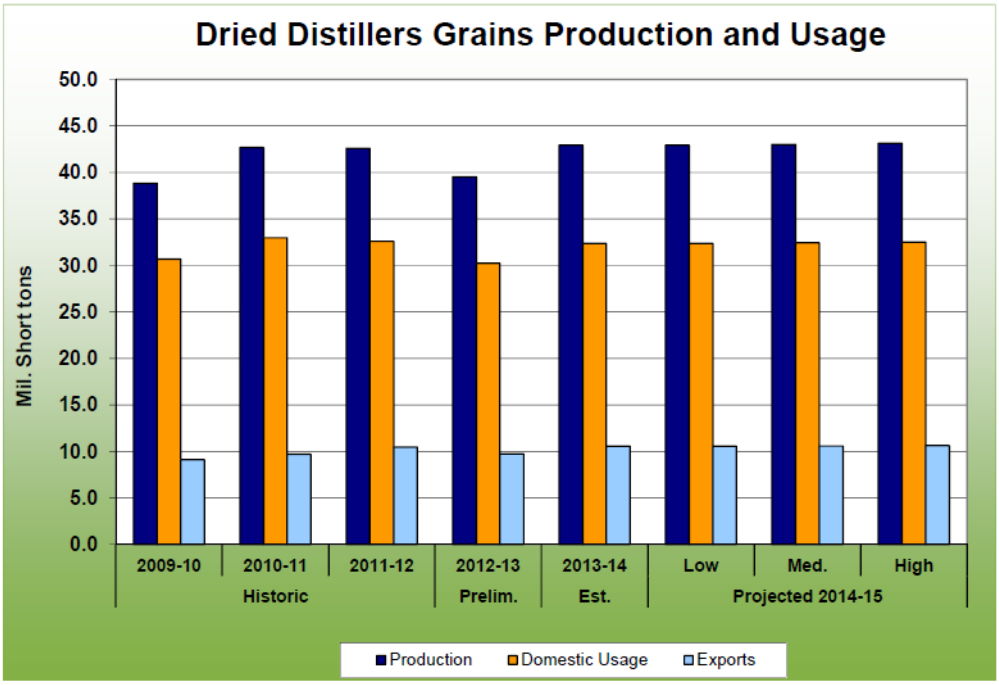


Figure 2-1. Dried Distillers Grains Production and Usage [6]

DDGS Production

Corn is the primary grain used in ethanol production because of its high content of fermentable starch compared to other feed stocks, as corn contains about 62% starch, 3.8% corn oil, 8.0% protein and 11.2% fiber, and 15% moisture [2]. The corn kernel has three components: the endosperm, germ and bran (tip and pericarp). The endosperm is mainly composed of starch, whereas the germ (about 12% of the kernel) is high in oil, protein, ash and non-fermentable carbohydrates. The bran portion is almost exclusively composed of dietary fiber, such as cellulose and hemicellulose [7].

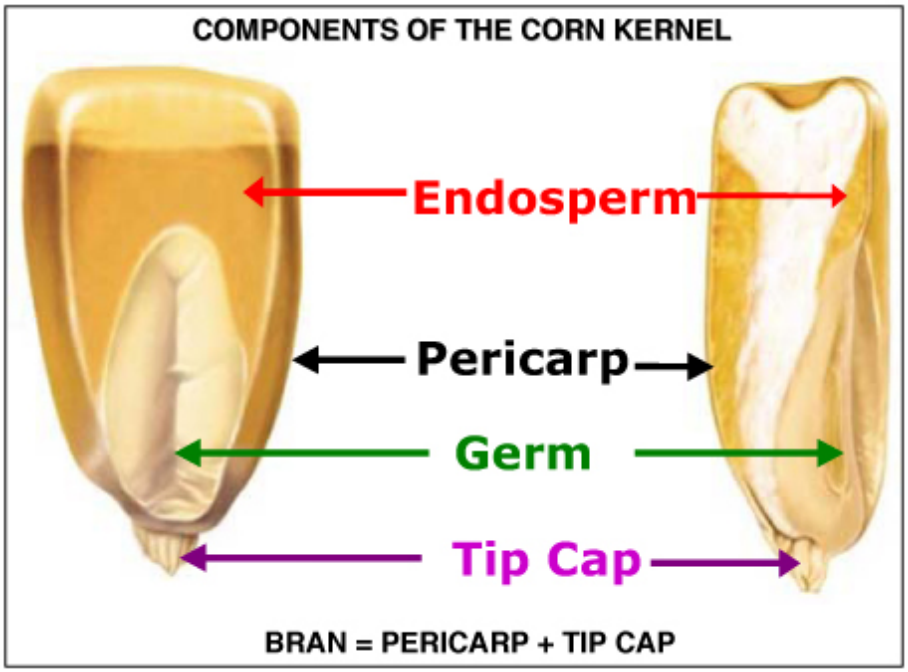


Figure 2-2. Corn structure

(Picture from http://pedagog.hubpages.com/hub/Why-Pop-corns-Pop# [8])

Two methods can be used to ferment corn into ethanol: wet milling and dry grinding. Most ethanol plants in the United States are dry-grind facilities. In 2009, dry grind plants represented 79% of all facilities [9]. In dry grind processing, each bushel of corn (25.4 kg) produces about 11.8 liters of ethanol and 7.7 kg of DDGS and 7kg CO_2 [10].

In dry grind plants, the hammer mill is used in the first step to reduce the particle size of corn. The starch is then hydrolyzed into glucose by adding amylolytic enzymes, and metabolized to ethanol by adding yeast. After fermentation, ethanol is collected by distillation. The remaining liquid and solids after distillation of ethanol are called whole stillage. The coarse solids are separated from the liquid by centrifugation. The liquid, called thin stillage, goes through evaporation, resulting in condensed distiller's solubles, which may be sold locally to cattle feeders or combined with the coarse solids fraction and dried to produce dried distiller's grains with solubles. The coarse solids contain about 35% dry matter and may be sold to local cattle feeders without drying, or dried to produce dried distiller's grains, or mixed with condensed distiller's solubles [1]. Figure 2-3 shows a schematic of the process of producing the various co-products resulting from the fermentation of corn.

To be mentioned, usually corn oil is separated out prior to fermentation, so as to increase the number and value of co-products from the dry grind ethanol process [11]. Meanwhile, the protein content of DDGS could be increased by removing the oil, making it a more valuable feed component [12]. Additionally, the high content of oil in DDGS limits its use in the diets of some animals, such as swine and cattle [13], so a modified

DDGS with lower fat and higher protein is desirable to meet the market need for DDGS in non-ruminant diets. Oil extracted from DDGS can be sold as a feedstock for biodiesel production, or as an edible oil for human food market if produced in food grade environment [14].

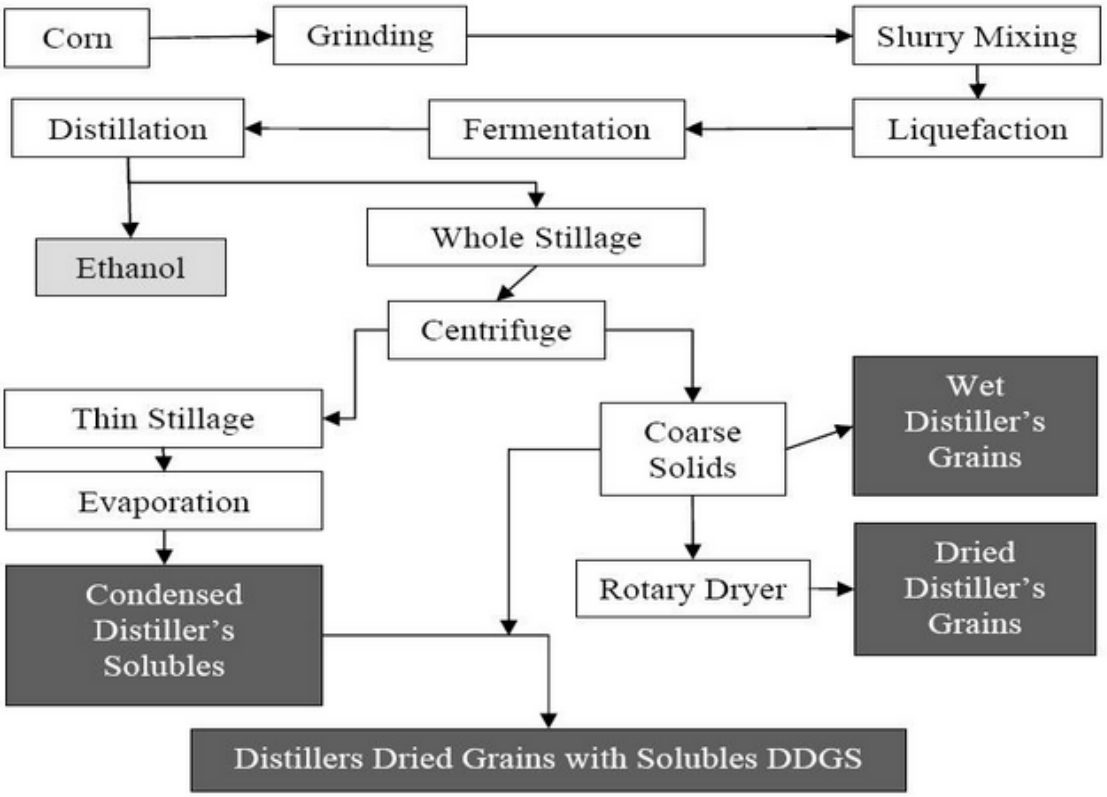


Figure 2-3. Dry-grind ethanol production process and co-products [15]

Within the past several decades, the United States has experienced a rapid growth in corn ethanol production. Figure 2-4 shows U.S. annual production of ethanol from grains and its main co-products, distillers dried grains with soluble (DDGS), between 1980 and 2010 [16]. In 2013, the United States ethanol industry produced 45.7 billion liters of distillers grains [2].

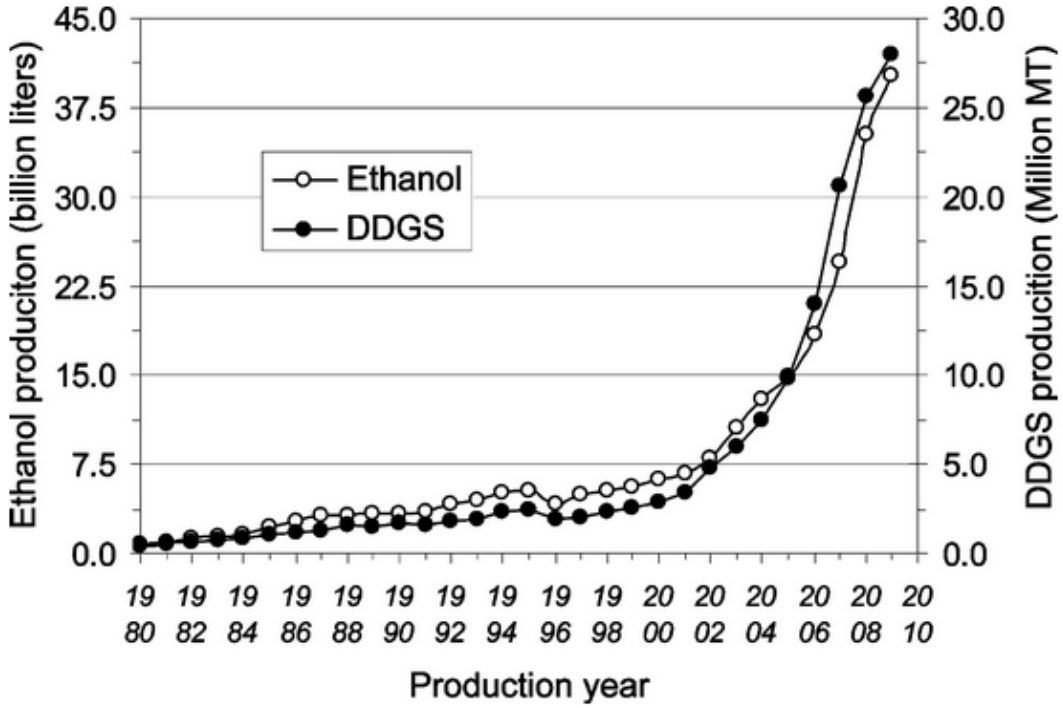


Figure 2-4. DDGS and ethanol production from 1980 to 2010 [17]

Composition of DDGS

DDGS has a similar nutritional profile to corn, except for the starch content. Belyea et al. [18] analyzed 235 DDGS samples from a fuel-ethanol plant in Minnesota and found that the average values (as % dry matter) for protein, oil, ash, crude fiber (mostly cellulose and hemicellulose), and ADF (acid detergent fiber) were 31.4, 12.0, 4.6, 10.2, and 16.8, respectively. They also reported the average content of residual starch to be 5.3%. Due to the different processing procedures, the compositional fractions in DDGS vary greatly, as shown in Table 2-1[19]. In 2008, Kim et al. [20] analyzed the DDGS components as: dry matter 88.8%, crude protein 24.9%, total carbohydrate 59%, glycan 21.2% (cellulose 16%, starch 5.2%), xylan 8.2% and arabinan 5.3%.

The main lipid component of corn is triacylglycerol, which is mostly stored in the germ. Minor lipids include phytosterols and tocopherols. The oil content of dried distiller grains with solubles really depend on if the oil extraction procedure is included in production [21, 22]. But linoleic acid is the major fatty acid (53.96%-56.53%), followed by oleic acid (25.2%-27.15%) and palmitic acid (13.25%-16.41%), with low levels of stearic (1.8%-2.34%) and linolenic (1.15%-1.40%) acid in corn oil [23]. Since fats can disrupt the rumen fermentation system, attention needs be paid when adding DDGS into ruminant diets [24].

Another valuable component in DDGS is xanthophylls, which are oxygenated carotenoids. However they can be lost under conditions of high heat, such as during drying of DDGS. Roberson [25] reported differences in xanthophyll content in two DDGS samples: one sample contained around 30 ppm of xanthophyll, while another one

had only 3.5 ppm. The difference was speculated to be due to the drying procedure and conditions. The NRC (National Research Council) [26] reported xanthophyll content in corn DDGS as 10.62 mg/kg, whereas 34 mg/kg was reported by Sauvant and Tran [27]. Drying condition is the predominate reason affecting the xanthophyll content of DDGS dramatically, since xanthophylls are very sensitive to heat.

Table 2-1. Range of values for DDGS composition

DDGS components	Range of values (% by wt.)[1]	Kim et al. [2] (% by wt.)
Dry matter	88-92	88
Crude protein	25-30	24.9
Total carbohydrate	55-60	59
Crude fat	8-10	NA[3]
Cellulose	14-18	16
Phosphorus	0.59-0.95	NA[3]
Crude fiber	6.0-7.0	NA[3]

[1.] Data from studies of Belyea et al., Batal et al. and Robinson et al. [18, 28, 29].

[2.] Data from studies of Kim et al. [20].

[3.] NA: Not available

Traditional usage of DDGS as animals feeds

The high nutritional content and the lower price of DDGS compared to other feed ingredients makes it a very attractive partial replacement for some of the traditional ingredients used in animal feeds. Domesticated animals fed DDGS-containing diets include poultry, swine, and cattle, although DDGS is also fed to fish, sheep, dogs, and horses [15].

Numerous studies have examined the effect of incorporating DDGS into animal feeds on growth and performance. Whitney and Shurson [30] showed there was no difference in % carcass lean or muscle quality characteristics of pork carcasses in pigs fed increasing levels of DDGS up to 30%. Another study indicated DDGS was successfully fed to laying hens at levels up to 10% without adverse effects on laying performance [31]. A similar study showed there were no negative effects of corn DDGS addition on the hens' feed consumption rate, egg production or interior egg quality, although egg weight decreased with increasing corn DDGS content up to 20% [32]. However, there may be circumstances when the dietary concentration of DDGS must be reduced. For example, one study indicated that 30% concentrations of DDGS fed to pigs early in the nursery phase might negatively affect growth performance [33].

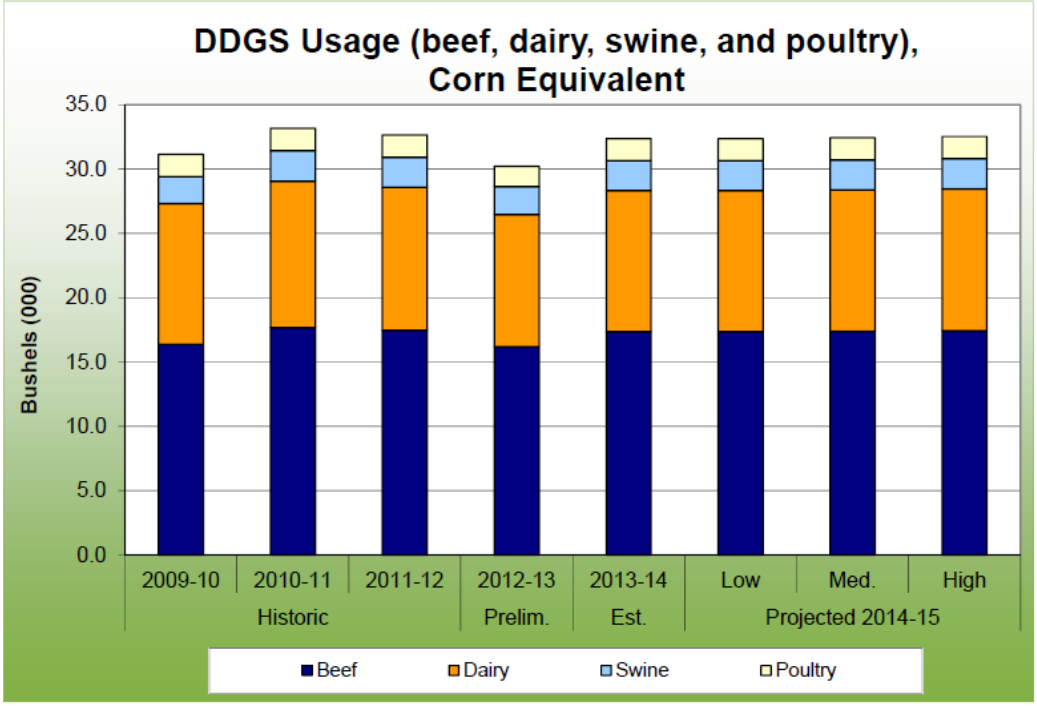


Figure 2-5. DDGS usage as a feed for domesticated animals [6]

DDGS as a food ingredient

Several studies examined the use of DDG as a substitute for flour or an additive in baked products, such as biscuits, cookies and batters [34-36]. By adding DDGS, the fiber and protein content of the baked products can be increased. This low starch, high protein and high fiber grains could be used as an ideal food ingredient for consumers with medical conditions such as diabetes or Celiac's disease [37]. Foods high in starch may increase blood glucose levels beyond the desirable range, but diets whole grain or high fiber may attenuate this increase, and are thus recommended to diabetes patients. As for people with celiac disease, when they eat gluten, their body mounts an immune response that attacks the small intestine, leading to damage on the villi and then nutrients cannot be absorbed properly into the body. Food made of DDGS would solve this problem because corn starch is gluten free.

The studies showed that DDGS can be used as a flour substitute up to about 15%, or in some cases even 25%, without producing an objectionable appearance or texture [38]. A major problem is that DDGS has a distinct odor and taste which might negatively affect acceptability for human foods. Addition of DDGS generally results in darker-colored finished food products. Bleaching and de-odoring of DDGS will be essential to its use in human foods to minimize the darker color development. Hydrogen peroxide, ethanol/ butanol extraction and lipoxygenases appear to be promising bleaching agents that should be pursued [39]. Additionally, the property of foods with added DDGS, such as expanding volume, moisture absorption, texture, and mouth-feel, would be altered as well [40].

Therefore, it appears that DDGS can be added into human foods at the level of 15% to 25%, depending upon the composition of the DDGS itself, and upon the specific food product [41]. Also, the use of DDGS depends upon the other ingredients in the food matrix, which might react with DDGS components and thus produce specific texture or flavor [42].

Dietary fiber

There is no single accepted definition of dietary fiber, but commonly the term dietary fiber refers to plant carbohydrates that are neither digested nor absorbed in the stomach and small intestine in human beings. Dietary fiber can be categorized in several ways, such as on the basis of its sources, structure, properties, or its application. One common classification of dietary fiber is as either soluble or insoluble. Soluble fiber includes fructans, inulin, pectin, β-glucans, and glucomannans, while insoluble fiber includes cellulose, and some arabinoxylans. The Academy of Nutrition and Dietetics [43] has recommended a total dietary fiber intake of 20 g/ day to 35 g/day for adults. Interest in dietary fiber continues to grow, because many epidemiological studies have shown that conditions such as diabetes, atherosclerosis, breast cancer, hemorrhoids, and obesity are associated with a low intake of fiber in humans [44].

Many benefits of diets containing high levels of dietary fiber have been promoted. The intrinsic physical properties of dietary fiber could explain dietary fiber's beneficial effects, such as bulking, gel formation, and viscosity change of gastric contents, modulation of gastric motor function, and fermentation [45]. For example, high fiber diets can provide lower caloric intake, and a decrease in body weight gain, because fiber-rich foods usually have a low energy density [43]. In the stomach, some of soluble fibers absorb large quantities of water, which may increase stomach distention. This may trigger afferent vagal signals of fullness, thereby contributing to satiety during meals and satiation in the post meal period [46]. Another well-known physiological effect of dietary fiber is its laxative effect. Both highly fermentable and poorly fermentable fibers increase

fecal mass in the colon. Poorly fermentable fibers such as cellulose produce a much larger fecal mass, which are usually more effective at producing laxation.

Soluble dietary fiber

The American Association of Cereal Chemists has defined soluble fiber as “the edible parts of plants or similar carbohydrates resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine” [47]. Most soluble fibers form viscous solutions when mixed with water and are readily fermented by gut microflora. The viscous solutions may have several important physiological effects, such as slowing intestinal digestion, thereby reducing postprandial blood glucose concentrations, or delaying the emptying of stomach and prolonging the feeling of fullness, both of which could contribute to weight control. Slower stomach emptying may also decrease blood glucose levels and increase insulin sensitivity, which help control diabetes [48-50].

Soluble fibers are also reported to decrease serum cholesterol concentration [51-53]. Several mechanisms for the hypocholesterolemic effect include decreases in cholesterol absorption, large intestinal fermentation, and increased bile acid excretion [54-58]. The intestinal fermentation of dietary fiber may change gut microflora profile and increase beneficial gut microflora (57).

Insoluble dietary fiber

Insoluble fibers accelerate movement of food through the small intestine. They are considered as gut-healthy fiber because they have laxative and bulking effects,

helping to prevent constipation [59]. However, compared with soluble fiber, which has been consistently demonstrated to produce moderate but significant reductions in cholesterol in animals and humans, insoluble fibers do not share the cholesterol-lowing effect [60]. However, certain insoluble fibers can be metabolically fermented in the large intestine [61].

Dietary fiber fermentation

Certain types of indigestible carbohydrates, including certain dietary fibers, undergo fermentation in the colon by microbes. The cecum (in rats) and proximal colon (in humans) are the primary sites of colonic fermentation. Colonic fermentation yields both gases and short-chain fatty acids (SCFA), such as acetic, propionic and butyric acids, which lower the pH of the contents [62]. The total amount of SCFA in the proximal colon is present in the range of 70 to 140 mM, and decreases to 20 to 70 mM in the distal colon. Therefore, the pH is lowest in the proximal colon and increases distally [62, 63].

The concentration of SCFA in the colon changes with the type of fiber and the location within the colon [64, 65]. Increases in SCFA decrease pH, which indirectly influences the composition of the colonic microflora. Short chain fatty acids facilitate the uptake of electrolytes and water [66] and also function to increase absorption of minerals, reduce ammonia absorption [67, 68], decrease solubility of bile acids, regulate glucose absorption, and influence immune functions [69-71].

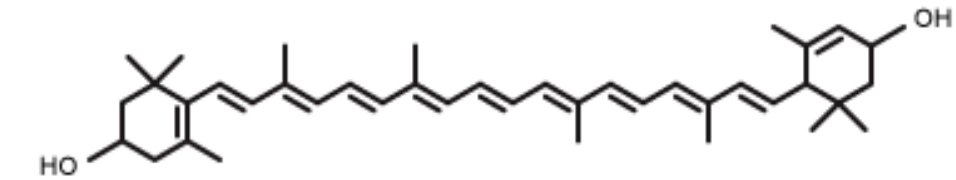
Carotenoids

Carotenoids are a group of natural lipophilic pigments synthesized by higher plants, some yeast, and fungi. Based on their chemical structure of the presence or absence of an oxygen atom in the molecule, carotenoids have two subclasses: if at least one oxygen is present, they are called xanthophylls; otherwise they are termed carotenes. The primary dietary carotenoids present in human plasma are lycopene, β-carotene, α-carotene, β-cryptoxanthin, γ-carotene, phytofluene, phytoene, lutein, and zeaxanthin. Lutein and zeaxanthin are reported to be major pigments in the human retina [72].

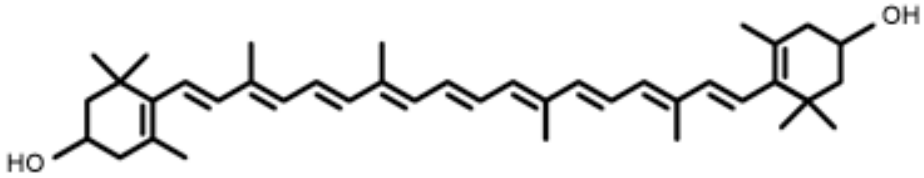
The corn kernel and corn oil are rich in carotenoids, especially lutein and zeaxanthin. Corn DDGS is reported to contain 66 μg/g oil of carotenoids, mainly composed of β-carotene, lutein, zeaxanthin, and β-cryptoxanthin [73]. The carotenoid content in the DDG oil was 33 times higher than reported in an oil extracted from corn germ using hexane, 5.5 fold higher than in hexane extracted corn fiber oil, and 2.5-fold higher than found in hexane extracted ground corn [73].

Lutein and zeaxanthin introduction

Lutein and zeaxanthin belong to the xanthophyll family of carotenoids and are the two major carotenoids in corn. Because they are not converted in the human body into retinol, they do not have pro-vitamin A activity. The difference between lutein and zeaxanthin is in the ionone ring: lutein contains a β-ionone ring and a ε-ionone ring, whereas zeaxanthin has two β-ionone rings [72]. Their chemical structures are shown below.



Lutein



Zeaxanthin

Figure 2-6. Lutein and zeaxanthin chemical structure

Lutein and zeaxanthin metabolism and bioavailability: influencing factors

In order to impart potential beneficial health effects, xanthophylls must be efficiently absorbed from food and carried to the target tissues. As discussed below, there are four major steps during the absorption of xanthophylls through the mucosal cells into the lymphatic system [74].

First, xanthophylls must be released from their food matrix. In some types of food matrix, the cell walls in raw vegetables could inhibit the release of xanthophylls. But processing and heat treatment of food can disrupt the cell wall structure to help increase the release of carotenoids. The food processing steps include grinding, fermentation, and mild heating, which could increase xanthophylls release by weakening the cell wall of plant tissues, dissociating the protein-carotenoid complexes, and dissolving the crystalline carotenoid complexes [75].

The second step of absorption is the transfer of xanthophylls to lipid micelles in the small intestines. The presence of dietary fat in the small intestine is required in this step to stimulate the gallbladder to release bile. Released xanthophylls then must be assimilated into the mixed lipid micelles in the lumen of the small intestine. Solubilized xanthophylls in the mixed micelle will be taken up by the intestinal epithelial cells [76].

The third step is to be taken up by intestinal mucosal cells. It is reported that xanthophylls passively diffuse through the cell membrane and are released into the enterocyte [77]. However, recent studies have suggested that the carotenoid uptake is partly mediated by facilitated diffusion [78].

The final step in xanthophylls absorption is the transport of them into the lymph system. In the Golgi apparatus of the enterocyte, xanthophylls are incorporated into chylomicrons. The chylomicrons are secreted into the lacteals, and are finally delivered to the systemic circulation. In the circulation, chylomicrons lose triglyceride content and shrink in size through the action of lipoprotein lipase on the endothelial lining, becoming chylomicron remnants. Eventually, the liver takes up the chylomicron remnants, which contain the absorbed xanthophylls. Xanthophylls either remain in the liver or are transported to the blood stream via secreted VLDL (very low density lipoprotein). Subsequently, xanthophylls (and other carotenoids) are taken up into tissues by the LDL (low density lipoprotein) receptor. Most lutein and zeaxanthin will specifically accumulate in the macula region of the eye [78].

The bioavailability of xanthophylls is thus influenced by several factors, including their chemical nature, as well as interaction of xanthophylls with other nutrients, and food processing [79]. The factors such as general malnutrition or intestinal parasites have been found to substantially reduce the efficiency of carotenoid absorption [79]. The bioavailability of lutein can also be reduced significantly when it is consumed with some forms of dietary fiber, including cellulose, wheat bran, guar and pectin [80, 81]. Dietary fat can positively influence the bioavailability of lutein [82]. This was illustrated in an experiment in which lutein, given as a diester supplement, showed a significant enhancement in absorption when given with a high dietary fat meal (the low fat meal contained 3 g fat, whereas the high fat meal contained 36 g of fat). Thus, fat was required for the sufficient solubilization of lutein diesters, as well as to stimulate secretion of

esterase and lipase from the pancreas to digest triglycerides, whicha are components of micelles.

Lutein and metabolites

Several proposed metabolites of lutein, whose structures are illustrated in Figure 2-7, were previously shown to be present in human plasma and tissues such as liver and retina [83]. 3′-Hydroxy-ε, ε-carotene-3-one and lutein were the predominant carotenoids in the plasma, liver, kidney, and adipose, accompanied by ε, ε-carotene-3, 3′-dione. 3-Hydroxy-β, ε-caroten-3′-one (3′-oxolutein) is the major metabolite of lutein in human plasma and the retina. 3′-Epilutein might be formed by a back reduction of 3′-oxolutein that was produced from lutein. Meso-zeaxanthin, which is detected in the retina only, might be formed by double bond migration from lutein [84, 85].

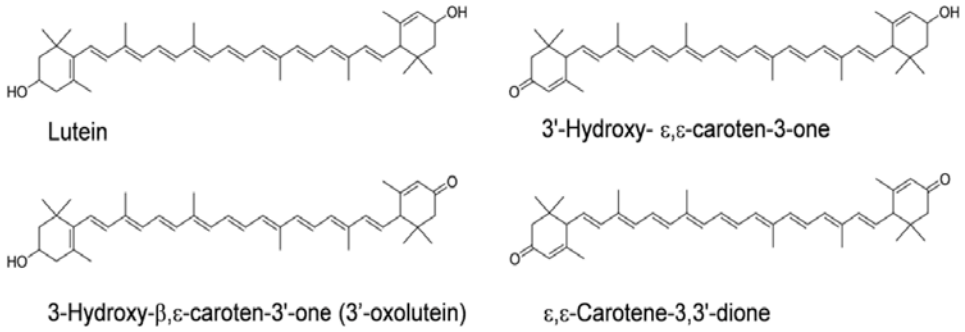


Figure 2-7. Lutein and metabolites

Potential health benefits

Lutein and zeaxanthin are reported to have biologically beneficial functions in decreasing cancer development, enhancing immune function and lymphocyte proliferation response [86], and protecting against age-related macular degeneration [87]. Total carotenoid intake is also associated with reduced risk of certain forms of cardiovascular disease, stroke, and cancer [88-90].

Several potential mechanisms have been reported to explain the protective effect of lutein against cardiovascular disease. Lipid peroxidation, a likely factor in the etiology of both retinal and cardiovascular disease, is inhibited by lutein and zeaxanthin (87). The presence of adhesion molecules on endothelial cell surfaces may lead to atherosclerosis pathogenesis. In-vitro research demonstrated that the incubation of lutein with cultured endothelial cells could effectively inhibit the expression of these adhesion molecules [91]. Other studies have found that lutein and zeaxanthin can inhibit thickening of the walls of carotid arteries and LDL-induced migration of monocytes into human artery cell walls [92]. Case-control studies measured atherosclerotic progression in asymptomatic men and women via carotid intima-media thickness (IMT) and have found a moderate inverse association between lutein and zeaxanthin serum levels and carotid IMT. In a three-year study, 231 asymptomatic age, sex, race, and field center-matched case subjects from the Atherosclerosis Risk in Communities (ARIC) study cohort demonstrated significantly lower serum lutein/zeaxanthin levels and an average IMT two times larger than matched control subjects [93]. Therefore, there is suggestive evidence that lutein and zeaxanthin may play a beneficial role in human health.

Age-related Macular Degeneration (AMD)

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness and visual impairment in older adults (>50 years) [94]. Age related macular degeneration typically results in a loss of vision in the center of the visual field as the area with the highest resolution. The damage to the retina makes it difficult or impossible to read or recognize faces, although enough peripheral vision remains to allow other activities of daily life.

There are two forms of AMD: dry and wet. The dry form of AMD is characterized by debris called drusen which accumulates between the retina and the choroid, eventually causing the retina to detach. The dry form of advanced AMD results from atrophy of the retinal pigment epithelial layer below the retina. The wet form of AMD is more severe: blood vessels expandfrom the choroid behind the retina, and the retina can also become detached. The risk factors leading to AMD include aging, family history, hypertension, and exposure to sunlight. Elevated cholesterol may also increase the risk of AMD [95]. Currently, AMD can be treated by laser coagulation and medication, which act to stop and sometimes reverses the growth of blood vessels [96].

The two major components of the macular pigment of the retina are lutein and zeaxanthin. These carotenoids may mitigate light-induced damage by filtering potentially harmful short-wavelength light [97]. Studies have shown that a lower concentration of retinal carotenoids was associated with increased macular diseases compared to controls [98, 99]. Therefore, vitamin supplements with high doses of lutein and zeaxanthin have

been suggested by the National Eye Institute and others to slow the progression of dry macular degeneration [100-102].

Lutein and zeaxanthin intake

Because of the potential role of lutein and zeaxanthin to improve human health, it is essential to boost the daily intake of lutein and zeaxanthin, which is currently low worldwide compared to what is considered optimal. The average daily intake of lutein in the United States is about 1.7 mg/day and in Europe it is about 2.2 mg/day [103]. Dietary sources are mainly from dark green vegetables such as spinach and kale, or from eggs. These values are below the level purported to reduce the risk of eye disease such as cataracts and AMD, since lutein supplementation at 10 mg/day and zeaxanthin supplementation at 2 mg/day are the amounts consumed that have been reported to decrease the risk of AMD [102]. However, xanthophylls amounts in these foods are not enough for our needs. Thus, the development of high-lutein staple foods would be an approach to increase lutein intakes.

Table 2-2 Foods with lutein and zeaxanthin [102]

Food	Serving	Lutein and zeaxanthin (mg)
Kale (cooked)	1 cup	23.8
Spinach (cooked)	1 cup	20.4
Collards (cooked)	1 cup	14.6
Corn (cooked)	1 cup	2.2
Broccoli (cooked)	1 cup	1.6
Eggs	2 (large)	0.3

Cholesterol

Cholesterol is an essential structural sterol component of animal cell membranes that serves to maintain proper membrane permeability and fluidity. Cholesterol also serves as a precursor for the biosynthesis of steroid hormones, bile acids, and the cutaneous synthesis of vitamin D. Cholesterol metabolism is tightly regulated. The whole-body cholesterol amount is controlled by the balance of cholesterol input (cholesterol absorption and de novo synthesis) and cholesterol elimination [bile acid synthesis and cholesterol (neutral sterol) excretion].

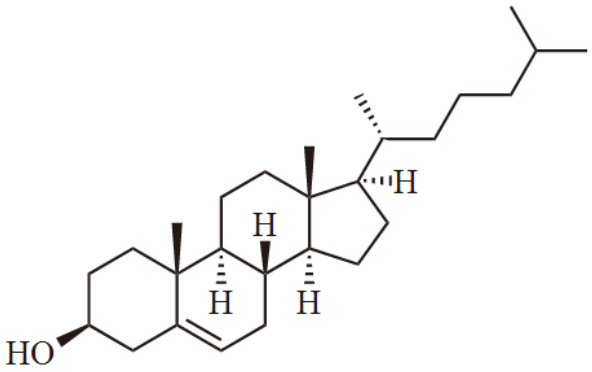


Figure 2-8. Cholesterol structure

Intracellular cholesterol regulation

The cholesterol concentration present in a cell regulates the rate of cholesterol biosynthesis directly. Normal healthy adults synthesize approximately 1 g/day of cholesterol and consume approximately 0.3 g/day. However, if the intake from food is high, then the endogenous production of cholesterol decreases. The cellular supply of cholesterol is maintained at a steady rate by three distinct mechanisms: 1. Regulation of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCoA-R) activity and levels; 2. Regulation of excess intracellular free cholesterol through esterification, by the activity of acyl-CoA cholesterol acyltransferase, ACAT; 3. Regulation of plasma cholesterol levels via LDL receptor-mediated uptake and HDL-mediated reverse transport [104, 105].

The primary way to control the rate of cholesterol biosynthesis is the regulation of HMGCoA-R activity. The enzyme is controlled by four distinct mechanisms: feed-back inhibition, control of gene expression, rate of enzyme degradation, and phosphorylation-dephosphorylation. In the first three regulation mechanisms, cholesterol acts as a feed-back inhibitor of pre-existing HMG-R as well as inducing rapid degradation of the enzyme. The latter is the result of cholesterol-induced polyubiquitination of HMGCoA-R and its degradation in the proteasome. Further, when intracellular cholesterol is in excess, the amount of mRNA for HMGCoA-R is reduced as a result of decreased expression of the gene [106, 107].

There are two main mechanisms for cholesterol output [108]. One is through the synthesis of bile acids. As bile acids are synthesized from cholesterol in the liver and secreted into the bile, the greater excretion forces the liver to continuously synthesize

new bile acids from stored cholesterol, thereby lowering circulating cholesterol. Meanwhile, cholesterol itself is also secreted in the bile. Unabsorbed cholesterol, either from the diet or from the bile, enters the colon, where some cholesterol is metabolized by bacteria flora to coprostanol (the principal sterol found in the feces), cholestenol, cholestanone, and epicoprostanol prior to excretion. Cholesterol and these bacterial metabolites are known as fecal neutral sterols, and their excretion is estimated to range between 350 to 900 milligrams per day, with cholesterol accounting for approximately 20% of the total [109]. The percentage of cholesterol converted to its bacterial metabolites has been shown to vary between populations [110]. In germfree rats, cholesterol is the only neutral sterol in the feces, demonstrating that the intestinal flora is responsible for the metabolism of cholesterol in the large intestine. The profile and metabolic activity of the microflora that metabolize cholesterol are influenced by dietary factors, including the type of diet and frequency of feeding [111].

Cholesterol absorption

Cholesterol enters the intestinal tract from two sources, the diet and bile, as stated above. The intestinal mucosa also may contribute a small amount. Biliary cholesterol is entirely unesterified, but a portion of dietary cholesterol may be esterified with fatty acids. Any cholesterol ester entering the intestine is de-esterified rapidly by pancreatic cholesterol esterase. Before absorption, cholesterol must be solubilized by micelles containing conjugated bile acids and hydrolytic products of triglycerides and lecithin - fatty acids, monoglycerides, and lysolecithin. Unesterified cholesterol is solubilized in the hydrophobic center of micelles. Cholesterol absorption occurs by micelles facilitating

its transport across the unstirred layer of water adjacent to the surface of the luminal cell [112].

Most dietary cholesterol is incorporated into chylomicrons as cholesterol esters in the intestinal mucosa. These lipoproteins contain mainly triglycerides that have been produced by the mucosa from newly absorbed fat. Chylomicrons are secreted into the lymph and eventually enter the systemic circulation. During interaction of chylomicrons with peripheral lipoprotein lipase, triglycerides are hydrolyzed to fatty acids and glycerol, producing a chylomicron remnant, but cholesterol remains within the remnant particle throughout lipolysis, until the chylomicron remnant is removed from the circulation by the liver. As cholesterol ester enters the liver within chylomicron remnants, its fatty acid apparently is released by an esterase. The resulting unesterified cholesterol can have four fates: re-esterified and stored as cholesterol ester; secreted into plasma with lipoproteins after re-esterification; converted into bile acids; or secreted into bile as cholesterol itself [113]. Factors influencing the efficiency of cholesterol absorption include: variation in cholesterol intake, the presence of plant sterols, the fiber content of the diet, transit time, and possibly the polyunsaturated to saturated fatty acid ratio of the diet [114, 115].

Niemann Pick C1 like (NPC1L1) is a recently discovered polytopic transmembrane protein, which functions as a sterol transporter to mediate intestinal cholesterol absorption and counterbalances hepatobiliary cholesterol excretion. It is essential for intestinal cholesterol absorption and transportation [116]. In cultured cells, NPC1L1 protein cycles between intracellular compartments and the cell membrane in a cholesterol-dependent manner. When cholesterol is high in cells, NPC1L1 is localized

predominantly in the endocytic-recycling compartment (ERC). When cholesterol is low in cells, NPC1L1 moves to the plasma membrane, so as to regulate cholesterol absorption. NPC1L1 transmembrane domains contain up to 5 sterol-sensing domains (SSD). These SSD are conserved in several polytopic transmembrane proteins and function in cholesterol metabolism and regulation, including NPC1, HMG-CoA reductase, SCAP [sterol regulatory element–binding protein (SREBP) cleavage-activating protein], and the Hedgehog signaling protein Patched. The SSD may regulate NPC1L1's intracellular itineraries by sensing membrane cholesterol content [117]. It has been suggested NPC1L1 may be transcriptionally regulated by cellular cholesterol content through SREBP-2, which is a membrane bound transcription factor that increases expression of cholesterol synthesis genes when cellular cholesterol content is low. It has been shown that variation in human NPC1L1 gene sequences contributes to reduced intestinal cholesterol absorption efficiencies and LDL-C levels [118].

Cholesterol transport

Lipids, including cholesterol, are transported by lipoproteins in the circulatory system. In addition to providing a means to solubilize cholesterol for transport through the blood, lipoproteins have cell-targeting signals that direct the lipids they carry to certain tissues. Chylomicrons, the least dense type of cholesterol transport particle, carry lipids from intestine to muscle and other tissues that need fatty acids. VLDL (very low density lipoprotein) particles are produced by the liver and export excess triacylglycerol and cholesterol that is not required by the liver for synthesis of bile acids. During transport in the bloodstream, lipoprotein lipase lining the blood vessels cleaves more

triacylglycerol from IDL molecules, which contain an even higher percentage of cholesterol. LDL (low density lipoprotein) particles are the major carriers of cholesterol in the blood. The shell of the LDL molecule contains just one molecule of apolipoprotein B100, which is recognized by the LDL receptor in peripheral tissues. HDL (high density lipoprotein) particles are thought to transport cholesterol back to the liver for excretion or utilization by other tissues after re-secretion in VLDL [119].

Dietary influence on cholesterol levels

The food one consumes not only influences cholesterol intake, but also can influence cholesterol synthesis. Dietary factors that influence human cholesterol synthesis include: energy restriction, meal frequency, dietary fat type (PUFAs increase synthesis), as well as cholesterol and phytosterol content [120].

Fat intake would have a difference influence on blood cholesterol levels depending on the type of fat. Isocalorically replacing dietary carbohydrates with monounsaturated and polyunsaturated fats has been shown to lower serum LDL and total cholesterol levels and increase serum HDL cholesterol levels, while replacing carbohydrate with saturated fat was shown to increase HDL, LDL and total cholesterol levels. Trans fats have been shown to reduce levels of HDL cholesterol while increasing levels of LDL cholesterol [121, 122]. Therefore, many health authorities advocate reducing LDL cholesterol through changes in diet and other lifestyle modifications. The USDA recommends that those wishing to reduce their cholesterol through a change in diet should aim to consume less than 7% of their daily energy needs from saturated fat and consume fewer than 200 mg of cholesterol per day [123].

Soluble fibers, such as β-glucans, have been shown to lower plasma LDL cholesterol [124-127]. The possible mechanisms for this effect is that increased viscosity in the small intestine can hinder the emulsion and interfere with the formation of small micelles, limiting the ability of the digestive tract to absorb all the cholesterol present [128, 129]. Insoluble fiber has not been found to have a significant cholesterol-lowering effect [130].

Abnormally high cholesterol levels (hypercholesterolemia), usually a combination of higher concentrations of LDL particles and lower concentrations of functional HDL particles, are strongly associated with cardiovascular disease because these promote atheroma development in arteries (atherosclerosis). This disease process leads to myocardial infarction (heart attack), stroke, and peripheral vascular disease. Details are discussed in the section on cardiovascular disease below [131, 132].

Bile acids

Bile acids are synthesized from cholesterol in the liver, which accounts for the majority of cholesterol breakdown in the body. Because of their ability to form micelles to solubilize and transport lipids in the aqueous environment of the intestinal lumen, bile acids play an essential role in lipid absorption. Approximately 95% of the bile acids are reabsorbed from the intestines, and the remainder is lost in the feces. The small fraction of bile acids which passes into the colon are largely converted into bacterial metabolites called secondary bile acids[133].

Primary and secondary bile acid metabolism is shown in Fig. 2-9 below [133]. Primary bile acids are synthesized in the liver from cholesterol via a series of reactions which convert the 27-carbon cholesterol molecule to a 24-carbon bile acid molecule containing α-OH groups on positions 3 and 7, and full saturation of the steroid nucleus. The 7α-hydroxylation of cholesterol is the first committed and rate limiting step in the pathway of bile acid synthesis. This reaction is catalyzed by the microsomal enzyme 7α-hydroxylase. The pathway of bile acid synthesis divides into two subpathways, one leading to cholic acid, the major primary bile acid family found in human bile, and another leading to chenodeoxycholic acid (CDCA). Once in the large intestine, bacteria further metabolize the majority of the primary bile acids to secondary bile acids through deconjugation and 7α-dehydroxylation of the bile acid molecule. Primary bile acids are more hydrophilic in nature, while secondary bile acids are more hydrophobic. Humans and hamsters have two primary bile acids, cholic acid (CA) and chenodeoxycholic acid

(CDCA), but several species have a third primary bile acid in addition to CA and CDCA. For example, the rat has α- and β-muricholic acids. Since bile acids are recycled through enterohepatic circulation, some biliary bile acids are secondary bile acids, which are formed from primary bile acids by bacteria enzymes in the colon. Deoxycholic acid (DCA) is derived from cholic acid and lithocholic acid (LCA) is formed from chenodeoxycholic acid [134].

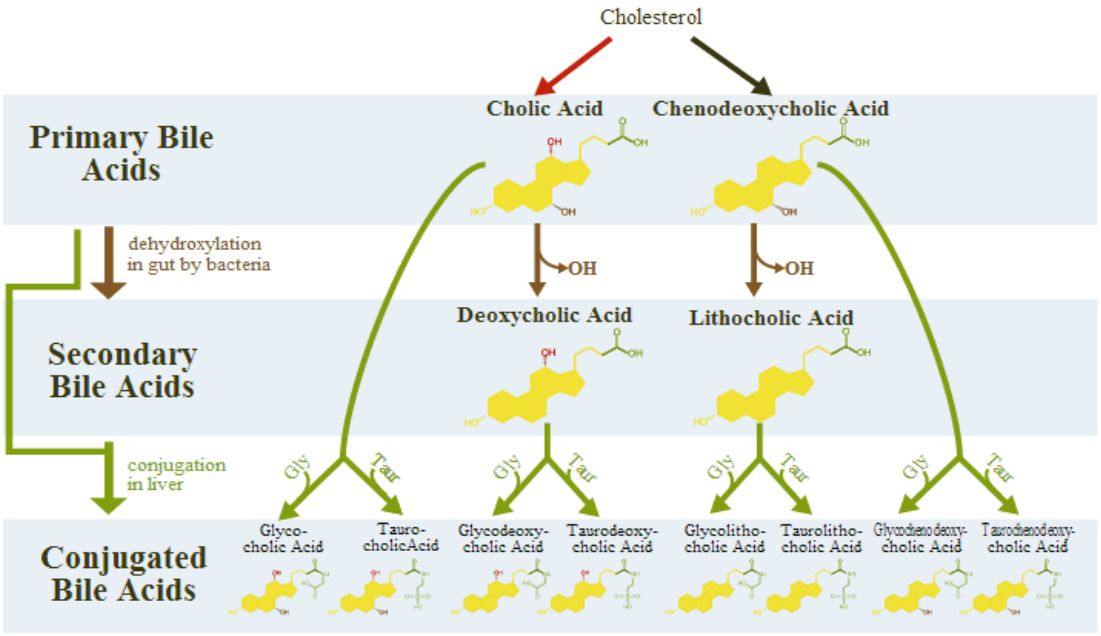


Figure 2-9. Bile acid metabolism [135]

Regulation of bile acid synthesis

Bile acids have the paradoxical role of contributing to both sides of the cholesterol input and output equation. They contribute to cholesterol input by promoting cholesterol absorption through incorporation of cholesterol into micelles in the small intestine. On the other hand, bile acids contribute to cholesterol output by being the major catabolic products of cholesterol metabolism and by inducing bile flow, which subsequently removes free cholesterol from the liver [134]. In addition to influencing cholesterol homeostasis, bile acids are involved in the solubilization and absorption of dietary fats and fat-soluble vitamins in the small intestine. Increasing dietary cholesterol increases synthesis of bile acids. So newly absorbed cholesterol could be transformed rapidly into bile acids. This mechanism may protect against the development of hypercholesterolemia [133].

Bile acids regulate cholesterol 7α-hydroxylase (CYP7A), the rate limiting step in bile acid synthesis, at the transcriptional level. In addition to the feedback control of bile acids and the feed-forward control of cholesterol, several hormones regulate cholesterol 7α-hydroxylase at the level of gene transcription [136]. Studies have shown that enzyme activity, mRNA levels and transcriptional activity of CYP7A all change in parallel in response to bile acid feeding or biliary diversion in vivo [137]. Primary bile acids are believed to be the mediators of negative feedback control on cholesterol 7α-hydroxylase. Also, two members of the nuclear hormone receptor family, LXRα and FXR, have been identified as the underlying mechanism for transcriptional regulation of the classic

feedforward and feedback pathways that control cholesterol and bile acid metabolism [138].

The effect of dietary cholesterol on cholesterol 7α-hydroxylase activity varies greatly depending on the species, and even within a species, which could contribute to the tremendous variation in plasma cholesterol concentrations observed both among different species and individuals of the same species. Dietary cholesterol has been found to increase transcription of cholesterol 7α-hydroxylase in the rat and mouse, but not in the hamster [139]. Plasma cholesterol levels in rats are virtually unaffected by large fluctuations in cholesterol intake or output. Rats have a 16 fold higher baseline 7α-hydroxylase activity on a cholesterol-free diet compared to the hamster, whose plasma cholesterol is very responsive to dietary cholesterol [140]. When given increasing levels of dietary cholesterol, both cholesterol 7α-hydroxylase activity and expression were markedly increased in the rat, but were not induced in the hamster. Perhaps the rat is not as susceptible to elevated plasma cholesterol levels because dietary cholesterol stimulates 7α-hydroxylase through feedforward activation to rapidly convert the extra cholesterol into bile acids, which does not seem to occur in the hamster. Humans fall somewhere in between these two extremes.

Effect of dietary fiber on fecal bile acid excretion

Dietary fiber has been shown to increase fecal bile acid excretion. It has been found that dietary wheat bran increased both total bile acid excretion and fecal weight without changes in fecal bile acid concentration [141]. The proportion of fecal hyodeoxycholic acid decreased with increasing dietary fiber, whereas that of lithocholic

and deoxycholic acids increased significantly with fiber intake. The concentration of fecal chenodeoxycholic acid did not change. Most studies have reported that diets high in any type of fiber lead to both an increase in the total excretion and a suppression in the bacterial degradation of bile acids, but the effect on fecal bile acid concentration appears to be less certain [142-145].

Binding of bile acids and increasing fecal excretion has been hypothesized as a possible mechanism for lowering cholesterol by dietary fiber [146]. By binding bile acids, cereal fibers prevent reabsorption and stimulate plasma and liver cholesterol conversion to additional bile acids [147, 148]. One study showed there were significant higher bile acids binding with rice bran and wheat bran fiber than with corn bran and cellulose [149].

Cardiovascular disease

Cardiovascular disease is any disease that affects the cardiovascular system, including cardiac disease, vascular diseases of the brain, and peripheral arterial disease. The causes of cardiovascular disease are diverse but atherosclerosis and/or hypertension are the most common reason [150]. Atherosclerosis is a specific form in which the artery wall thickens because of invasion and accumulation of white blood cells, containing both living active white blood cells and remnants of dead cells, as well as cholesterol and triglycerides, eventually calcium and other crystallized materials, within the outer-most and oldest plaque [151].

Cardiovascular disease is the leading cause of death worldwide, though, since the 1970s, cardiovascular mortality rates have declined in many high-income countries. At the same time, cardiovascular deaths and diseases have increased at a fast rate in low- and middle-income countries [152]. Although cardiovascular disease usually affects older adults, the antecedents of cardiovascular disease, notably atherosclerosis, begin in early life, making primary prevention efforts necessary from childhood. There is therefore increased emphasis on preventing atherosclerosis by modifying risk factors, such as healthy eating, exercise and avoidance of smoking tobacco. Dietary prevention recommendations includes a low-fat, high-fiber diet including whole grains and plenty of fresh fruit and vegetables [153] .

Coronary Heart Disease (CHD)

Coronary heart disease, also known as atherosclerotic heart disease, is the most common type of heart disease. The disease is caused by plaque building up along the inner walls of the arteries of the heart, which narrows the arteries and reduces blood flow to the heart.

Coronary heart disease continues to be the leading cause of death in the United States. Epidemiological, animal, and human studies have demonstrated that elevated levels of plasma cholesterol are an important risk factor for CHD [154, 155]. Individuals who have plasma cholesterol level greater than 240 mg/dL are at high risk for CHD, and a level less than 200 mg/dL is recommended. Many studies have shown that diet and lifestyle modification can reduce plasma cholesterol. Therefore, it is important to identify and encourage dietary practices that effectively lower cholesterol and are easy to incorporate into the diet [156, 157]. Research has shown oxidized low-density lipoprotein (LDL) to be a key factor in the initiation of atherosclerosis, one of the pathological processes involved in cardiovascular disease. Consequently, antioxidants, including carotenoids, have been investigated for the ability to scavenge free radicals and inhibit lipid peroxidation in cardiovascular and cerebrovascular disease [158].

Oxidative stress

Oxidative stress is a result of an imbalance between the production and disposal of reactive oxygen species (ROS) produced during metabolism. The ROS include free radicals, such as superoxide ($\cdot O_2^-$), hydroxyl ($\cdot OH$), peroxyl ($\cdot RO$), and hydroperoxyl

($\cdot HRO_2^-$), as well as non-radical species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl). These species can cause oxidation of proteins, lipids, and DNA and can also inhibit enzymes in the electron transport chain, blocking mitochondrial respiration and therefore diminishing normal cellular functions. All of these effects can cause cell injury, and may lead to cell death [159]. In recent years, it has become clear that oxidation of LDL is involved in many processes of atherogenesis, including the formation of cholesterol-engorged cells called foam cells [160]. Accumulation of foam cells (the fatty streak) in the subendothelial space damages the overlying endothelium, which allows platelets to aggregate and release powerful mitogens that contribute to further development of the lesion [158]. Therefore, oxidative stress appears to increase the risk of developing atherosclerosis.

Measurements of oxidative stress

A universally accepted, comprehensive measure of oxidative stress does not exist because of the changing concentrations of oxidation compounds produced throughout the peroxidation process. Furthermore, the amount and rate of production of various peroxidation products may depend on the unique fatty acid profile of various dietary lipids. There are two categories for analytical tests used routinely to assay lipid peroxidation: indicative tests and predictive tests.

Indicative tests measure products of peroxidation at a specific time to indicate the extent of peroxidation that has occurred. Examples of commonly utilized indicators include: peroxide value, conjugated dienes, thiobarbituric acid reactive substances, and

hexanal value. Alternatively, specific products of peroxidation such as HNE (4-hydroxynonenol) can be measured.

Generally, iodine value (IV) can indicate extent of peroxidation, as peroxidation causes the unsaturated fatty acid content to decline as PUFA are degraded. Additionally, the weight of lipid samples increases during peroxidation since oxygen incorporates into lipid hydroperoxides. However, these methods have limited usefulness in practical situations because they require compositional knowledge of the original (unperoxidized) lipid to ascertain the change made by peroxidation. Furthermore, these general measurements are not specific and require additional assays to identify specific products of peroxidation [161].

The peroxide value (PV) quantifies the concentration of peroxides and hydroperoxides in a lipid using an iodometric procedure with titration of sodium thiosulfate. However, this method has been criticized for being too subjective because the end point is determined by the analyst. Consequently, high performance liquid chromatography, and infrared spectroscopy are used to be objective as possible. But in practice, the utility of PV is limited when assessing the extent of lipid peroxidation because peroxides are simultaneously created and degraded during peroxidation. A low PV can be misleading because it may represent minimal peroxidation or that extensive peroxidation has occurred but that peroxides have been degraded [162]. Therefore, the practical utility of PV is limited without knowledge of historical values for a lipid.

The TBARS (thiobarbituric acid reactive substances) assay is used commonly to measure malondialdehyde (MDA), a secondary product of lipid peroxidation. In this

assay, one mole of MDA reacts with 2 moles of TBA under acidic conditions to form an adduct with a pink-red color and an absorption maximum at 530 to 532 nm. However, 2-alkenals and 2, 4-alkedienals, and other products of peroxidation react with TBA, so this assay is not specific to MDA. Furthermore, inter-laboratory comparisons of TBARS concentration is challenging because several methodological variants are employed. Like other indicative measures, TBARS follows a bell-shaped curve of production followed by degradation. As a result, changes in TBARS concentration can lead to misinterpretation of low values without knowledge of historical values for the lipid [162].

Predictive tests evaluate the ability of a lipid to withstand peroxidation when exposed to standardized, accelerated conditions to induce peroxidation. Routinely used predictive tests include the active oxygen method (AOM), oxygen radical absorbance capacity (ORAC), and ferric reducing antioxidant power (FRAP), which are used both in food systems and in vivo.

Study Aim

In summary, evidence from epidemiological and animal studies suggests that the components in DDGS, such as dietary fiber and xanthophylls, would have beneficial effects on health. However, currently no study has been conducted to test if DDGS could have these effects directly. Therefore, the aim of the following study was to examine the effects of DDGS and its co-products on cholesterol and bile acid metabolism, as well as xanthophylls bioavailability in rats.

Chapter 3: Materials and methods

Experimental design

A total of 40 male Wistar rats, 125-149 g body weight, were randomly divided into 4 groups, and housed individually in wire-bottom stainless steel cages. The temperature was controlled at 22º C with a 12 hour light-dark cycle. All experimental procedures were approved by the University of Minnesota Animal Care and Use Committee.

Rats were fed the American Institute of Nutrition (AIN-93G) purified diet for 5 days to adapt to the new living environment. The animals were fed either the AIN-93G (control) diet, DDGS diet, bran fraction diet, or DDGS-soluble fraction diet for 3 weeks. The diet composition is shown in Table 3-1. DDGS, bran fraction or DDGS soluble fraction were incorporated into their respective diets at a concentration of 20%. The macronutrient concentration was constant for all diets. Cornstarch was used to adjust total carbohydrate content, corn oil was used to adjust total fat, and cellulose was used to adjust total fiber. Animals were allowed free access to water and diets.

Body weight and food intake was measured each week. Food intake was determined by subtracting amount of food offered from diet remaining in the food cups after 24 hours, and spillage. After 14 days feeding, feces were collected to determine fecal bile acid and neutral sterol excretion. Urine was collected after 24 hour fasting for measurement of thiobarbituric reactive substances (TBARS).

At the end of the feeding period, rats were anesthetized using isoflurane. Blood was collected by cardiac puncture into tubes containing EDTA (1 mg/mL) and centrifuged at 1200*g* for 15 min at 4° C. Plasma samples were collected and stored at -80°C until analysis. Epididymal fat pads, cecum with contents, and livers were excised, weighed, flash frozen and stored at -80° C until analysis.

Table 3-1. Composition of diets

Diet Ingredient (g/kg)	AIN 93G Control	DDGS	Bran Fraction	DDGS-Soluble Fraction Dry
Product		*200*	*200*	200
Carbohydrate (50%)	500	500	500	500
Corn Starch	300.48	264.48	259.5	215.3
Dextrinized Corn Starch	115.2	115.2	115.2	115.2
Sucrose	87.3	87.3	87.3	87.3
Carbohydrate from product	*0*	*36*	*41*	*85.2*
Protein (20%)	200	200	200	200
Casein (>85% protein)	200	148.2	181.6	166.8
Protein from product	*0*	*51.8*	*18.44*	*33.2*
Fat (15%)	150	150	150	150
Oil (Corn)	150	124.4	133.2	112.2
Fat from product	*0*	*25.58*	*16.84*	*37.82*
0.15% Cholesterol	1.5	1.5	1.5	1.5
Total Fiber (9.5%)	95	95	95	95
Cellulose	95	39	1.2	66.81
Total fiber from product	*0*	*56*	*93.8*	*28.19*
Mineral Mix (93G-Mx)	35	35	35	35
Vitamin Mix (93G-Vx)	10	10	10	10
L-Cystine	3	3	3	3
Choline Bitartrate	2.5	2.5	2.5	2.5
TBHQ	0.014	0.014	0.014	0.014
Moisture	***0***	***26.14***	***27.52***	***0***
Ash	***0***	***8.36***	***5.58***	***21.2***
Total	1000.0	1000.0	1000.0	1000.0

Analytical Methods

Urinary TBARS

To determine oxidative stress, thiobarbituric acid reactive substances (TBARS) were measured in the urine. Assay of TBARS largely measures malondialdehyde (MDA) generated from lipid hydroperoxides. Urine samples were mixed with 5% TCA and 0.06M TBA solution and heated at 80° C for 90 minutes. After cooling, samples were centrifuged at 2000 rpm for 10 min, and absorbance of the supernatants measured at 535 nm [163].

Liver cholesterol

Lipids were extracted from approximately 1 g of liver tissue with 20 volumes of chloroform: methanol (2:1) by the method of Fölch et al. [164]. Samples were dried under nitrogen and solubilized with Triton X-100/acetone. Concentrations of total and free cholesterol were determined spectrophotometrically by an enzymatic method, using cholesterol oxidase, cholesterol esterase and peroxidase [165]. Absorbance was measured at 500 nm.

Bile acids assay

Bile acids were extracted from 100 mg feces using ethanol and chloroform: methanol (2:1) and purified by solid phase extraction using C18 columns [166]. The eluates were collected and evaporated with nitrogen gas and stored in the freezer until ready to analyze.The bile acid extracts were assayed enzymatically by the method of [167] using 3α-hydroxysteroid dehydrogenase. Absorbance was monitored at 340 nm.

Neutral sterol excretion

Fecal lipids were extracted into chloroform:methanol (2:1, vol/vol) and saponified. Non-saponifiable sterols were extracted into hexane, purified by washing with KOH and water, and dried under nitrogen. Sterol were dissolved in pyridine and derivatized with Sylon BTZ. An aliquot (1µl) was injected onto a gas chromatograph equipped with a 12 m x 0.2 mm (i.d.) HP1 column and a flame-ionization detector. Hydrogen was used as a carrier gas at a pressure of 40 kPa. Sterols were calculated from the peak areas relative to the peak area of the internal standard, 5a-cholestane. Differences in detector response among the various compounds were corrected on the basis of the response factors calculated from a mixture of pure steroids with known molar composition.

Xanthophylls extraction from diets

An aliquot of each co-product-containing diet was mixed with 40% methanolic KOH solution at 56° C for 20 min. After cooling, samples were placed in the dark for 1 hour. More hexane was added to the mixture and the upper layer was collected and dried under nitrogen, avoiding light. The dried extract was then dissolved in 1 mL mobile phase A [methanol, methyl- tert- butyl- ether (MTBE) and water, 60:33:7] for analysis by LC-MS.

Xanthophylls extraction from plasma and liver

Liver samples were homogenized with 9 parts ice-cold isotonic saline using a homogenizer and used for extraction of lutein and its metabolites. In the case of liver, the

extracts were saponified separately with 2 mL of 10 mM KOH at 60° C for 45 min before extraction of lutein and its metabolites. These operations were done under dim yellow light to minimize isomerization and oxidation of carotenoids by light.

Dichloromethane:methanol (2:1, v/v) was added to plasma samples to extract xanthophylls. More hexane was also added to mixture. The extract was pooled, evaporated to dryness using a stream of nitrogen and finally dissolve in mobile phase A for LC-MS [84].

HPLC analysis of xanthophylls

High performance liquid chromatography (HPLC) was employed initially to identify xanthophylls in the co-products and rat plasma and liver. Mobile phase A contained methanol, methyl-tert-butyl-ether (MTBE) and water in a ratio of 60:33:7 by volume and mobile phase B in a ratio of 8:90:2. A Bischoff C30 column was used for separation. UV detection was at 455 nm [168].

The gradient used was 100% mobile phase A for 3 min, 15 min linear gradient to 80% mobile phase A; 17 min linear gradient to 55% mobile phase B; 15 min linear gradient to 95% mobile phase B, 2 min gradient back to 100% mobile phase A. The flow rate was 0.8 mL/min.

LC-MS analysis of xanthophylls

Xanthophylls were also analyzed by APCI-LC-MS at the Center for Mass Spectrometry and Proteomics on the St. Paul campus. UV detector wavelength was set at 455 nm. The parameters of the APCI source were source heater temperature as 475° C, sheath gas flow rate (arb) was 80, and Aux gas flow rate (arb) as 10, discharge currents as 5.00 µA, capillary temperature as 225° C, capillary voltage as 19 V, and tube lens offset as -25V. As for the ion optics, multiple 1 offset as – 4V, lens voltage as -24V, multiple 2 offset as -7.5 V, and multiple RF amplitude as 400V.

Statistical analysis

All results were analyzed by one-way analysis of variance using SAS 9.3 (SAS institute, Cary, NC). Differences among means were inspected using Duncan's multiple range test. A P-value less than 0.05 was considered statistically significant.

Chapter 4: Results

Body weight, food intake, epididymal fat pads

Initial body weights did not differ among the dietary treatment groups. Weekly body weight and food intake are shown in Table 4-1. At the end of 3 weeks of feeding, there were no significant differences in food intake or body weight. As a measure of adiposity, epididymal fat pads were weighed, and there were no significant differences in epididymal fat pad weights among the groups.

Cecum pH and fecal weight

Cecal pH is an indicator of fermentability of the diets. There were no significant differences in cecal pH among the groups. Cecum weight (with contents) in the bran fraction diet and the control diet group was significantly greater than in the soluble fraction and DDGS diet groups. Rats in the soluble fraction group had a significantly lower dry weight of feces than rats in the control group, but the DDGS diet group and bran fraction diet group did not differ significantly from either the soluble fraction or control group diets.

Urinary thiobarbituric acid reactive substances (TBARS)

Urinary 24 h TBARS was measured as a marker of whole body lipid peroxidation. There were no significant differences in daily urinary TBARS excretion among the 4 diet groups.

Liver weight, liver cholesterol and fecal bile acids

Liver weight did not differ significantly among the diet groups. Liver cholesterol concentration, however, was reduced in all co-product diet groups compared to the control diet group. The groups fed DDGS and the soluble fraction diets had the lowest liver cholesterol concentration, and did not differ from each other. The bran fraction diet was intermediate between the control diet and DDGS and soluble fraction diets.

As bile acids are synthesized from cholesterol, an increase in bile acid excretion could be responsible for the reduction in liver cholesterol by diverting more cholesterol into bile acid synthesis to replace bile acids lost in the feces. The three co-product diet groups all had greater bile acid excretion compared to control group. The greatest excretion was in the bran fraction diet, followed by the DDGS and soluble fraction diets, which did not differ from each other. Fecal bile acid excretion correlated somewhat with the reduction in liver cholesterol concentration ($r=-0.39$, $p=0.013$), and thus may partly explain the reduction in liver cholesterol. However, given the modest correlation, there are likely additional mechanisms involved in the reduction in liver cholesterol by the co-products diets.

Fecal Neutral Sterols

Fecal neutral sterols are cholesterol and its bacterial metabolites. Table 4-2 shows the daily fecal sterol excretion for each diet group. Coprostanol and cholesterol were the major neutral sterols present, with only relatively small amounts of dihydrocholesterol found. There was a significantly greater excretion of total neutral sterols excretion from the group fed the diet containing DDGS than the group fed with diet containing bran fraction. The control diet group showed a significantly greater ratio of cholesterol to total

neutral sterols, followed by the DDGS and bran fraction diet groups, with the soluble fraction diet group showing the lowest ratio.

In addition, total steroid excretion was calculated as the sum of total bile acids and total neutral sterol: results are shown in Table 4-3. Groups fed with DDGS and bran fraction diet groups showed significantly highest total steroid excretion, followed by soluble fraction diet group, and the control group had the lowest amount. This result is consistent with steroid balance between food intake cholesterol and steroid excretion. The control group had significantly highest steroid balance amount, which would lead to more cholesterol accumulation in liver. However, the negative average balance in bran fraction diet group would contribute to decreased liver cholesterol concentration theoretically.

Xanthophylls in diets and tissues

The xanthophyll concentration of the DDGS and other co-products used in the diets was determined by HPLC, and is shown in Table 4-4. A representative HPLC chromatogram of the standards is shown in Fig 4-4 and a chromatogram of the xanthophylls in DDGS is shown in Fig 4-5. Lutein was present in a greater concentration than zeaxanthin in all three co-products. The soluble fraction had the greatest concentration of xanthophylls, with DDGS only slightly less. The bran fraction had a relatively low concentration of xanthophylls.

Measurement by HPLC of the xanthophyll concentration in plasma of rats was attempted. Lutein and zeaxanthin could be detected in the plasma of rats two hours after gavage with a concentrated extract of xanthophylls obtained from marigold flowers (Fig 4-7). However, repeated attempts to detect xanthophylls by HPLC in the plasma of rats

two hours after a meal of a DDGS-containing diet were unsuccessful (Fig 4-6), which was apparently due to the much lower concentration of xanthophylls in the plasma.

Next, LC-MS was employed as a more sensitive technique for measuring xanthophylls in animals fed co-product-containing diets. Initially, an LC-MS system using an electrospray ionization (ESI) source was utilized, based on literature reports indicating successful use of this type of source [169, 170]. However, detection of standards was never achieved, in spite of use of several different types of mobile phase dopants reported to improve ionization, and therefore detection, of xanthophylls [170].

Atmospheric pressure chemical ionization (APCI) is another method for ionizing compounds of interest for mass spectrometry that has been used successfully for quantitating xanthophylls [170, 171]. Using an LC-MS system equipped with an APCI source, the xanthophylls standards lutein and zeaxanthin were both successfully detected (Fig 4-9). In the positive APCI LC-MS mode, the most abundant molecules are m/z 569 (lutein/zeaxanthin +H) and m/z 551 (lutein/ zeaxanthin +H-H_2O). The retention time for lutein and zeaxanthin are 11.66 min and 13.88 min respectively.

Using this APCI-LC-MS system, attempts were then made to detect xanthophylls in the plasma, liver, and feces of animals fed the co-product-containing diets. However, repeated attempts to do so were unsuccessful.

Chapter 5: Discussion

Currently, DDGS, generated from U.S ethanol industry, is primarily used as a feed for livestock and poultry [2]. However, DDGS has potential use as a human food ingredient, based on its excellent nutritional qualities. As corn is exceptionally rich in lutein, and products such as DDGS and the DDGS soluble fraction are promising ingredients in the development of high-lutein functional foods. Here we examined whether DDGS and related products may have human health benefits, based on its high concentration of xanthophylls, antioxidants, and dietary fiber. Positive findings of benefits related to human health would likely increase the value of these co-products to the ethanol industry.

The corn bran fraction diet was studied as a fiber control group since it contains almost the same fiber contents as DDGS, but the method used to remove starch from these two products was different. A physical method was used to remove starch from corn bran, whereas an enzymatic method was used in DDGS, that is, enzymes expressed by yeast during fermentation.

After 3 weeks consuming the diets, there were no significant differences in body weight, food intake or fat pad weights among the groups, indicating that the co-products were well tolerated by the animals when fed at 20% of the diet. These results are consistent with the study conducted by Whitney and Shurson [172], who found that no difference in weight gain in pigs when fed diets containing 20% DDGS, compared to a control group.

Two indicators of intestinal fermentation were examined in this present study, cecal pH and cecal weight with content. With increased fermentation, cecal pH is reduced due to the increased production of short-chain fatty acids. Cecal pH showed no significant difference among the diet groups, suggesting a lack of an increase in fermentation by the co-products. However, cecal weight in the DDGS and soluble fraction diet groups was significantly greater than the control diet and bran fraction diet groups. Since it is well established that increases in fermentation increase cecal wall weight [173, 174], this result suggests that DDGS and soluble fraction co-products may increase intestinal fermentation. These two co-products contain as a fiber source arabinoxylans, which have been shown to be fermentable and lead to an accumulation of short-chain fatty acids [175]. In contrast, cellulose, which is highly resistant to fermentation, was the only fiber present in the control diet. Interestingly, the bran fraction diet, which contained virtually no added cellulose, did not increase cecal weight. Thus, the fiber present in this corn bran appears to be resistant to fermentation, consistent with previous studies in both humans [176] and rats [177]. Another, more indirect way to assess fermentation is by analyzing the profile of fecal neutral sterols. A change in the profile of neutral sterols towards a greater proportion of bacterial metabolites of cholesterol may also indicate changes in microfloral populations or greater microbial activity. In rats fed diets containing DDGS, bran fraction, or DDGS soluble fraction, more of the cholesterol in the feces was metabolized into coprostanol and dihydrocholesterol, compared to the control group. This is indirect evidence that all the co-products influenced the colonic microflora. Determining how the microfloral

populations changed with consumption of the co-products may warrant further investigation.

Urinary 24 h TBARS excretion was measured as a marker of whole body lipid peroxidation. A reduction in urinary TBARS is typically associated with a lessening of oxidative stress [162]. In this study, there were no significant differences in urinary TBARS excretion among the four diet groups. However, there were several aspects of the diets that could influence oxidation state in our study. On the one hand, DDGS from corn contains a relatively large amount of polyunsaturated fatty acids (PUFA), especially linoleic acid (18: 2), and some yeast components, which may increase oxidative stress [13]. On the other hand, DDGS is rich in antioxidants, such as ferulic acid and carotenoids [178, 179]. However, since the control diet was formulated with corn oil, all diets would have had similar amounts of PUFA, and thus this factor would seem to be equalized among the groups.

Ferulic acid (FA) is a phenolic acid that present in corn in a high concentration. FA is an antioxidant which neutralizes free radicals such as superoxide, nitric oxide, and hydroxyl radical, all of which may cause oxidative damage to cell membranes and DNA [180]. However, FA is mostly bound to the cell wall in corn and has low bioavailability due to limited release by gut microbiota [181]. In the present study, the small amount of FA that was likely absorbed [182] is unlikely to have a meaningful effect on overall antioxidant status.

Another antioxidant component is the xanthophylls, which may reduce the incidence of age-related macular degeneration (AMD), cancer, and coronary heart disease

[183, 184]. The mechanism of action is thought to be due to the ability of xanthophylls to act as an antioxidant or to filter harmful short-wave blue light in the macula [185]. Lutein may act as an antioxidant due to its centrally localized conjugated C=C double bonds, which can readily quench reactive oxygen species. Additionally, hydroxyl groups attached to each of the two terminal β-ionone rings make lutein more efficient at scavenging free radicals than other carotenoids. Our hypothesis was that the xanthophylls contributed by the co-product diets would lower oxidative stress due to their antioxidant activity. However, the reason for the failure to see a reduction in urinary TBARS may be that the xanthophylls were not well absorbed, since they could not be detected in plasma or tissues by our methods, as discussed below. Further, our animal model was a healthy rat without any treatment to increase their oxidative stress, which may have made it difficult to detect an antioxidant effect. Finally, whether xanthophylls are functioning as antioxidants in vivo is still uncertain [186-188] . Evidence suggesting a lack of antioxidant activity by xanthophylls was provided by Collins et al. [189], who treated volunteers with a variety of carotenoids, including lutein. Their results showed that carotenoids supplementation did not have a significant effect on endogenous oxidative damage.

One more point to be considered is that TBARS of the diets were not measured in our study. This may be important, as suggested by a study in pigs in which there was 21% greater value for dietary TBARS in the DDGS diet compared to the control diet. After feeding a diet containing 35% DDGS for 38 days, there were no differences in basal circulating indicators of oxidative stress between the DDGS and control groups, but

urinary TBARS were increased in pigs fed with DDGS [190]. It was suggested that the greater urinary TBARS excretion may have been due to the 21% greater TBARS in the DDGS diet compared to the control diet. The lack of greater urinary TBARS with co-product feeding in our study may have been due to using co-products in which the lipids were not oxidized. And as we mentioned in introduction part, TBARS assay is a good indicator for oxidative stress, but it cannot comprehensively illustrate the oxidative stress, because changes in TBARS concentration can lead to misinterpretation of low values without knowledge of historical values for the lipid.

Cholesterol homeostasis is maintained through the coordination of several input and output pathways. The cholesterol input pathways include de novo biosynthesis by liver and other tissues, including the small intestine, and the absorption of dietary cholesterol. The output pathways include secretion of free cholesterol into the bile and conversion of cholesterol to bile acids [191]. In this study, all rats had the same cholesterol intake since all four diets contained 0.15% cholesterol, and since there was no significant differences in their food intake, cholesterol intake would have been the same in all diet groups.

All groups fed diets containing co-products had lower liver cholesterol concentration compared to the control group. Of the co-products, the groups fed the diets containing DDGS or DDGS soluble fraction showed the lowest liver cholesterol concentration. Further, the three co-product diet groups had greater bile acids excretion than the control group. Therefore, co-products did show a health benefit of lowering liver cholesterol, which likely involved increasing bile acid excretion.

What components of the co-products may have been responsible for causing the reduction in liver cholesterol is uncertain. Greater cholesterol output may have been caused by arabinoxylans [192]. Arabinoxylans, a soluble dietary fiber extracted from corn bran, could reduce the level of liver cholesterol. Consumption of water-soluble fiber and some insoluble fibers has been shown to decrease cholesterol levels [193]. Three major mechanisms have been suggested to explain the cholesterol-reducing effects of soluble dietary fiber: prevention of bile salt re-absorption from the small intestine, reduced glycemic response leading to lower insulin stimulation of hepatic cholesterol synthesis, and physiological effects of fermentation products of soluble dietary fiber, mainly propionate [194]. In vitro studies have shown that intake of some soluble dietary fiber results in 35-65% excess bile acids excretion in the feces [195, 196], which might be due to decreased bile salts reabsorption into the enterohepatic circulation. Viscous fiber could also reduce the rate of intestinal absorption of glucose with subsequent decrease in insulin production by the pancreas. The reduced insulin levels could lead to a decrease in cholesterol synthesis [197, 198]. However, the fiber present in the co-products is unlikely to produce viscosity within the small intestinal lumen, and thus fiber viscosity seems unlikely to play a role in the cholesterol lowering in our study. In addition, dietary fiber causes an increase in short chain fatty acids (SCFA), especially propionate, which may indirectly cause a decrease in blood cholesterol via inhibition of hepatic cholesterol synthesis [199, 200].

In our study, the group fed the DDGS diet showed lowest total liver cholesterol, but not the highest total bile acids excretion. Therefore, increased bile acid excretion may

not be sufficient to account for the observed cholesterol reduction. We thus considered another cholesterol output, fecal neutral sterol excretion. The DDGS diet group showed the greatest total fecal neutral sterol excretion, while the bran fraction diet group was the lowest. However, these differences were not statistically different. Lopez et al. reported that rats fed a diet containing about 8% arabinoxylans had significantly greater neutral sterol excretion, compared to the control group, but no difference in bile acid excretion [192], the opposite of our findings. On the other hand, total steroid excretion, which is the sum of bile acids and neutral sterol, were greatest in the DDGS and bran fraction groups. Furthermore, all co-products diets had a much lower total steroid balance, which is the balance between cholesterol from food intake and total steroid excretion, compared to the control group. These results suggest that the reduction in liver cholesterol concentration by DDGS and the soluble fraction co-product may have been due to greater steroid excretion, at least in part.

The DDGS soluble fraction diet contained the greatest amount of lutein and zeaxanthin, 2.11μg/g diet, while the DDGS diet contained the second greatest amount, at 1.69 μg/g diet. However, no lutein or zeaxanthin was detected in plasma or liver of the rats, even using LC-MS. It appears that the level of these xanthophylls in plasma were too low to be detected, possibly due to metabolic transformations such as formation of conjugates by detoxification enzymes after the intestinal uptake [201]. Another reason for this result might be poor xanthophylls bioavailability from diets. According to the literature, lutein absorption requires the presence of dietary fat, which stimulates the release of emulsifying bile acids by the gallbladder [202]. Rats fed a low-fat diet fail to

absorb xanthophylls efficiently, whereas they do so when the diet contains 10% fat. However, this point seems to not explain our findings, since the diets we used contained 15% fat. Another reason might be dietary fiber, especially insoluble fiber [203], which has been suggested to have a negative effect on carotenoid absorption by entrapping carotenoids. Xanthophylls might also have been metabolized. One study [85] showed that after 14 days of lutein ester supplementation, 3'-hydroxy-ε, ε-caroten-3-one (with molecular weight 549, 567) and lutein (with molecular weight 551, 569) were the predominant carotenoids in plasma and tissues, accompanied by ε, ε-carotene-3, 3'-dione (with molecular weight 565). Lutein and its metabolites accumulated most in the liver, followed by plasma, adipose tissue and kidney. This result was consistent with studies in humans [204, 205]. Further, they acknowledged that lutein was the predominant carotenoid found in mice after dietary supplement in other studies [85, 206], while the detailed amounts of other detected metabolites were not shown. Nevertheless, neither lutein nor its metabolites were detected in our study by LC-MS.

In conclusion, the consumption of diets containing 20% DDGS or its co-product solubles fraction appeared to increase large intestinal fermentation, based on enlargement of the cecum and a change in the profile of neutral sterols towards a greater proportion of bacterial metabolites. These two diets also resulted in a lower liver cholesterol concentration, which may be explained in part by an increased bile acids excretion. However, no increase was found in fecal neutral sterol excretion. Whole body oxidative stress was not changed in any of the diet groups. This result might be due to both PUFA and antioxidants content in DDGS. Unfortunately, xanthophylls were not detectable in plasma or tissues. Based on the effect of lowering liver cholesterol, these co-products of bioethanol production warrant further examination as a food supplement providing health benefits for humans.

Advantages and limitations of the study and future directions

This study has many advantages, such as a study design using several control groups and a diet composition that was carefully balanced for macronutrients. The AIN-93G purified rodent diet was used as control diet. Additionally, the corn bran fraction diet was used as the fiber control group, to compare the different effects of two different starch removal methods: a physical milling method for the bran fraction diet, and a chemical and enzymatic method for the DDGS diet. Further, this is the first study to examine the potential health benefits of DDGS in rats model to our knowledge. However, there are also some limitations. One limitation is that DDGS tested in our study only comes from one source. The composition of DDGS varies significantly, depending on the production practices, as discussed in the literature review. It is possible that the findings

may vary depending on where and how DDGS was produced. In addition, the concentration of DDGS in the diet was arbitrarily set as 20%, but a different concentration would possible make a difference. Finally, as with all animal studies, to what degree the results can be extrapolated to human is uncertain.

A future research direction should be to study xanthophyll accumulation in the retina, as studies show that the retina is one of the tissues where there is the greatest accumulation of xanthophylls. A second area of further research would be to determine bacterial metabolites of xanthophylls, which are currently unknown, in order to understand better the bioavailability of xanthophylls. Finally, further research is needed to study the mechanism by which DDGS lowers liver cholesterol. In particular, examining the effect of DDGS on endogenous cholesterol synthesis is warranted.

Table 4-1 Measures of body weight gain and tissue weight

Parameter	Control	DDGS	Bran Fraction	Soluble Fraction
Body weight gain (g)	152.4±7.0	148.6±7.3	144.9 ±7.0	138.0 ±9.4
Epididymal fat pads (g)	3.2±0.3	3.2±0.2	3.2 ±0.2	2.8 ±0.2
Cecum pH	6.5±0.1	6.6±0.1	6.6 ±0.0	6.6 ±0.1
Cecum weight with content (g)	2.5±0.1[a]	3.3±0.2[b]	2.6±0.1[a]	3.5±0.3[b]
Fecal dry weight (g/3 d)	7.1±1.2[a]	6.8±1.0[ab]	6.5±0.9[ab]	6.0±0.75[b]
Urinary TBARS[2] (μg/24h)	6.6±1.1	6.4±1.1	5.8 ±0.7	5.6±0.3
Liver weight (g)	16.9±1.2	15.1 ±0.7	16.1±0.9	15.3 ±1.1
Liver total cholesterol[3] (mg/g)	13.2±0.9[a]	7.2±0.7[b]	9.8±1.0[c]	7.0 ±0.8[b]
Bile acids[3] (mg/24h)	6.6±0.6[a]	23.9±1.6[b]	30.1±2.1[c]	17.3±0.6[d]

All results were expressed as least squares means ± SEM, n=10 per group. Data were analyzed by ANOVA. Values within a row that do not share a common superscript are significantly different, $p<0.05$.

[1.] Means with the different letter are significantly different.
[2.] Measured after 2 weeks of feeding of the diets.
[3.] Measured after 3 weeks of feeding of the diets.

Table 4-2 Daily Fecal Neutral Sterol Excretion

Neutral Sterols (μmol/24 hour)	Diet group			
	Control	DDGS	Bran Fraction	Soluble Fraction
Cholesterol	17.0±2.4[a]	13.8±1.4[ab]	11.1±1.3[ab]	9.1±1.8[b]
Coprostanol	8.5±0.8	13.7±1.7	12.3±1.7	14.8±2.7
Dihydrocholesterol	1.6±0.2	2.8±0.4	2.5±0.4	2.6±0.5
Total	27.0±2.9[ab]	30.3±3.3[a]	25.9±3.3[b]	26.5±4.8[ab]
Ratio of cholesterol to coprostanol+ dihydrocholesterol	1.8±0.8[a]	0.9±0.2[b]	0.8±0.2[c]	0.5±0.3[d]
Ratio of cholesterol to total neutral sterols	0.6±0.2[a]	0.5±0.1[b]	0.4±0.1[b]	0.3±0.1[c]

All results were expressed as least squares means ± SEM, n=10 per group. Data were analyzed by ANOVA. Values within a row that do not share a common superscript are significantly different, $p<0.05$.

Table 4-3 Total steroid excretion

Sterol excretion (μmol/ 24 hour)	Diet group			
	Control	DDGS	Bran Fraction	Soluble Fraction
Total bile acids	16.20±4.43 [a]	58.71±12.66[b]	73.65±15.91 [c]	42.44±4.43 [d]
Total neutral sterols	27.02±2.93[ab]	30.30±3.26[a]	25.88±3.26[b]	26.54±4.79[ab]
Total steroid excretion	43.23±9.83 [a]	89.00±17.11 [b]	99.512±22.44 [b]	69.98±17.35 [c]
Total steroid balance	40.16±8.94 [a]	3.53±17.00 [bcd]	-7.84±17.29 [c]	17.24±11.74 [d]

All results were expressed as least squares means ± SEM, n=10 per group. Data were analyzed by ANOVA. Values within a row that do not share a common superscript are significantly different, $p<0.05$.

Daily cholesterol intake= (3^{rd} week 24 hr food intake*0.15%/386.65) - 24hr total steroid excretion

Table 4-4 Xanthophylls concentration of the bioethanol co-products

	Lutein	Zeaxanthin	Total
	(μg/g)		
Control Diet	0.06	0	0.06
DDGS Diet	1.15	0.54	1.69
Bran Fraction Diet	0.27	0.14	0.41
Soluble Fraction Diet	1.51	0.60	2.11

All results were expressed as least squares means ± SEM, n=10 per group. Data were analyzed by ANOVA. Values within a row that do not share a common superscript are significantly different, $p<0.05$.

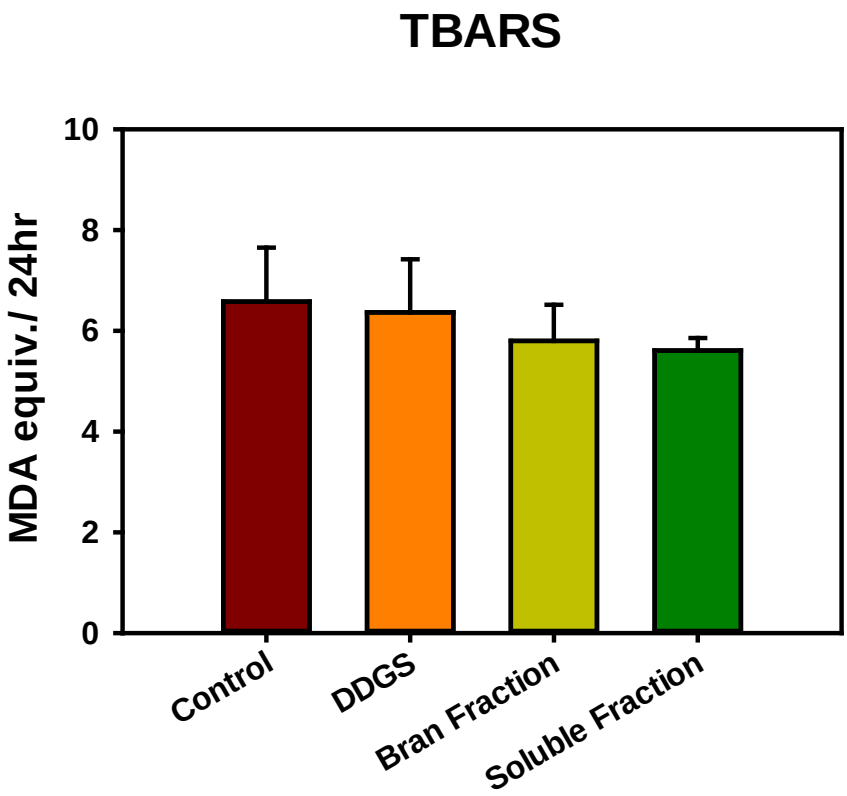


Figure 4-1 Urinary TBARS excretion over 24 hrs.

All results were expressed as least squares means ± SEM, n=10 per group. Data were analyzed by ANOVA. Values within a row that do not share a common superscript are significantly ifferent, $p<0.05$.

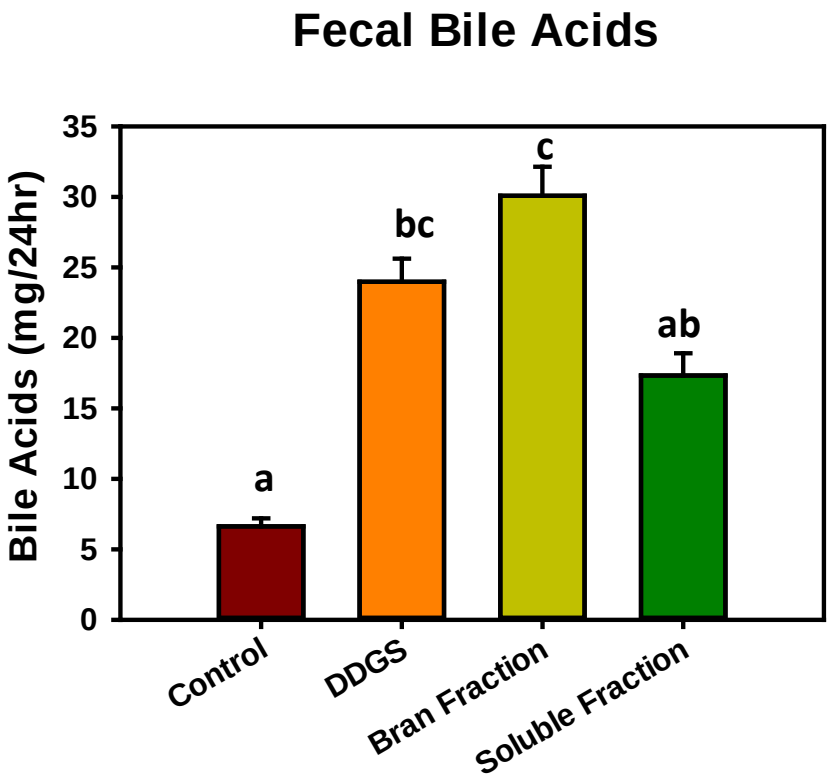


Figure 4-2 Bile acids excretion over 24 hrs among diet groups

All results were expressed as least squares means ± SEM, n=10 per group. Data were analyzed by ANOVA. Values within a row that do not share a common superscript are significantly different, $p<0.05$.

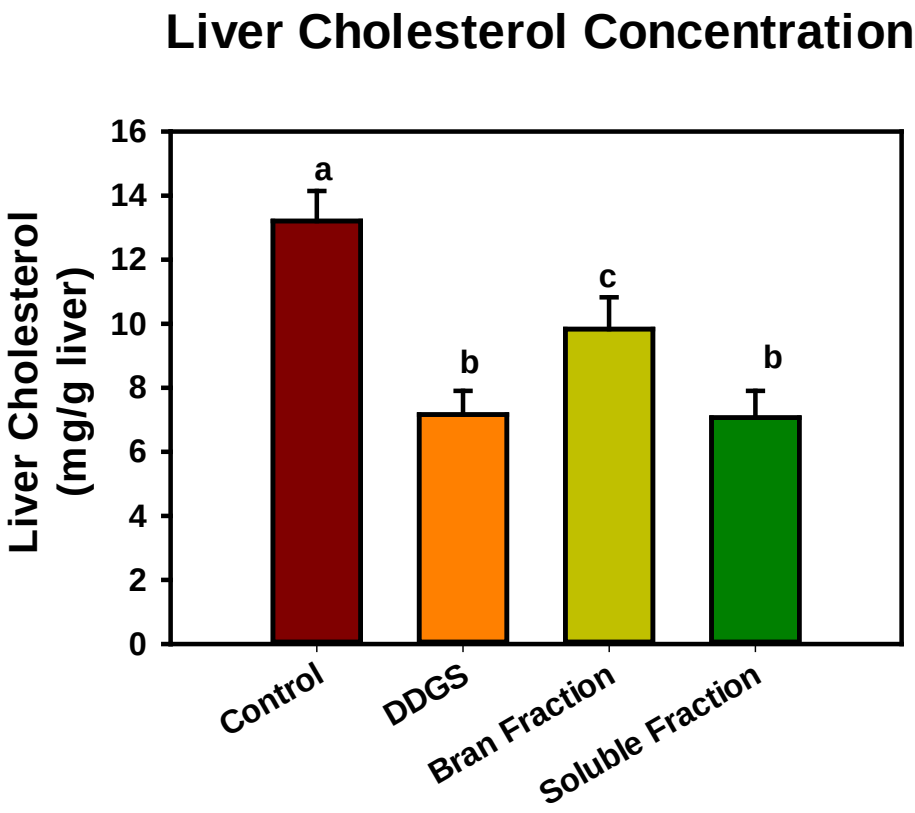


Figure 4-3 Liver cholesterol concentration

All results were expressed as least squares means ± SEM, n=10 per group. Data were analyzed by ANOVA. Values within a row that do not share a common superscript are significantly different, $p<0.05$.

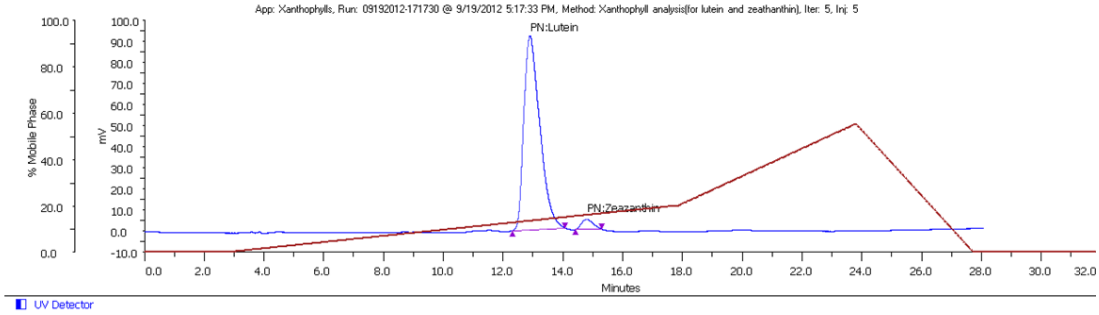


Figure 4-4 Lutein and zeaxanthin standard by HPLC

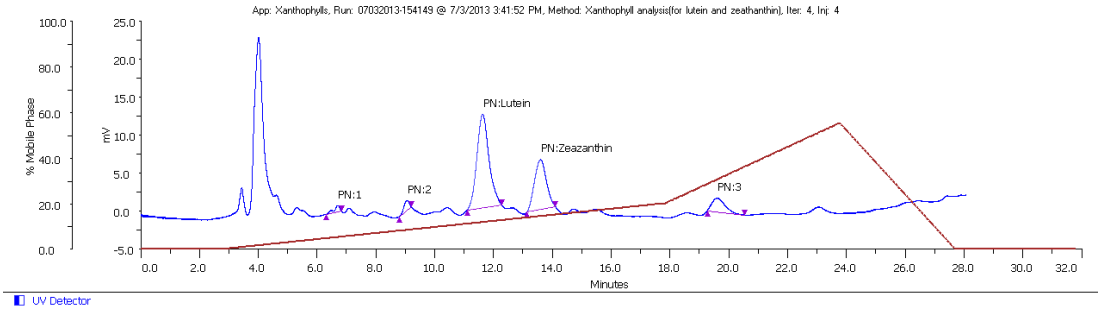


Figure 4-5 DDGS diet sample by HPLC

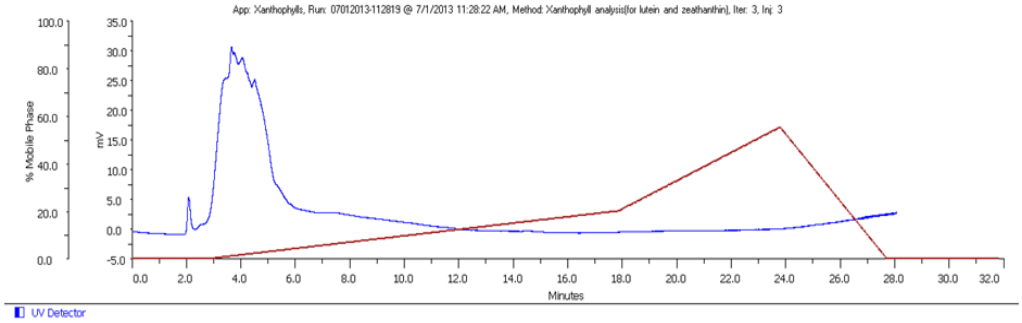


Figure 4-6 Rat plasma samples 2 hours after a meal of DDGS by HPLC

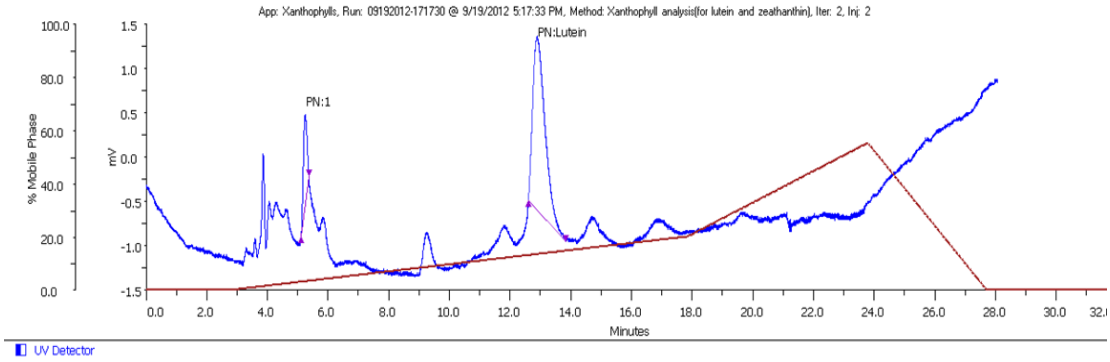


Figure 4-7 Rat plasma sample 2 hours after gavage with marigold extract solution by HPLC

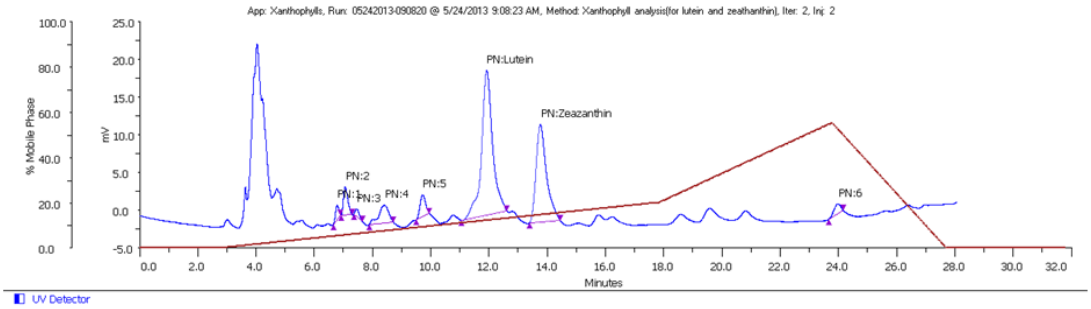


Figure 4-8 Marigold flower extraction solution in HPLC

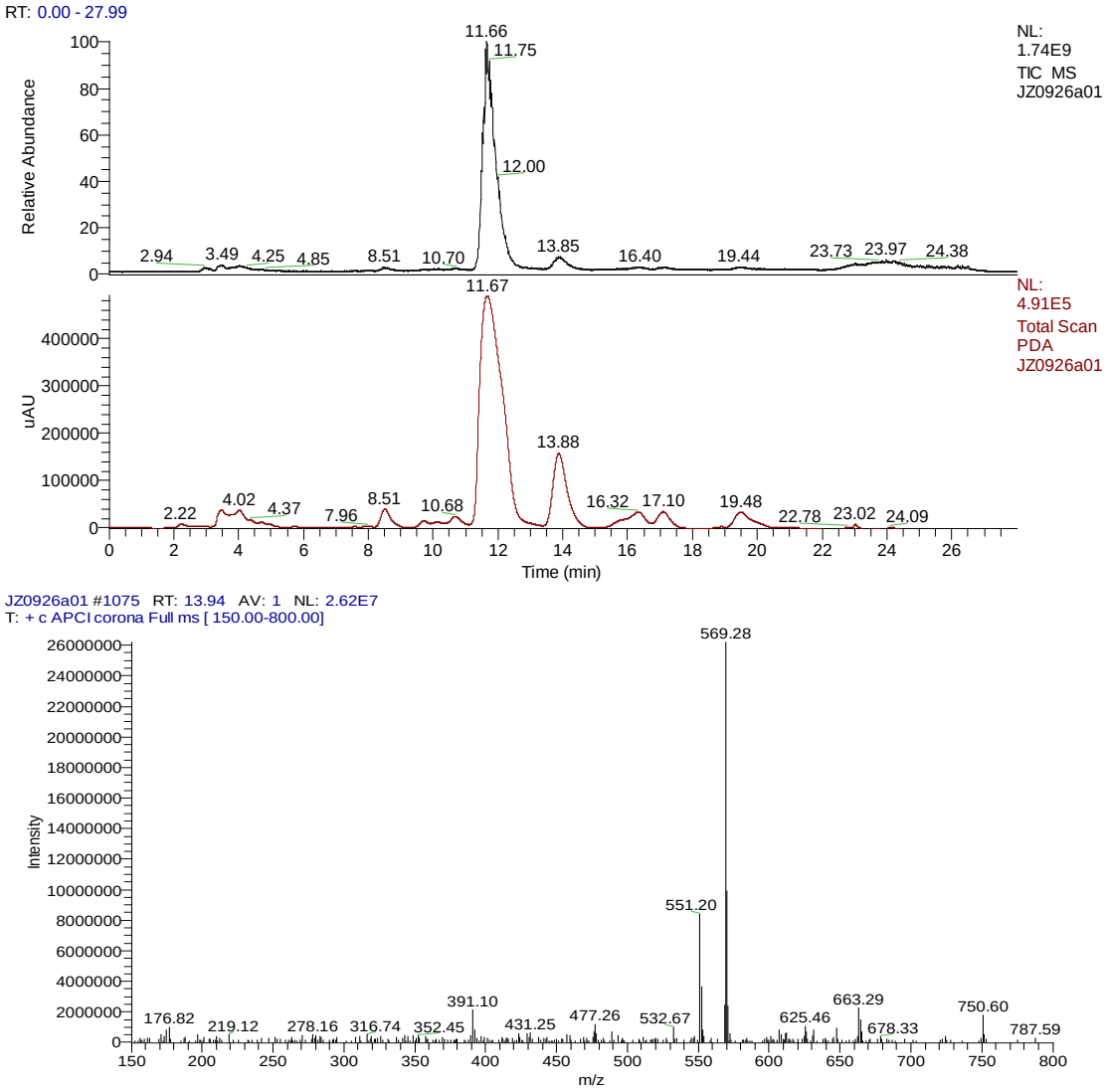


Figure 4-9 Lutein and zeaxanthin standards by LC-MS

References

1. *A Guide to Distiller's Dried Grains with Solubles (DDGS)*, r. Edition, Editor 2013, U.S Grains Councils.
2. Outlook, E.I., *2013 Ethanol Industry Outlook*, 2013, Renewable Fuels Association: Washington DC, USA.
3. Thong, L.A., et al., *Distillers Dried Grains with Solubles as a Supplemental Protein Source in Diets for Gestating Swine.* Journal of Animal Science, 1978. **46**(3): p. 674-677.
4. Parson, C.M., C. Martinez, V.Singh, and S.Noll, *Nutritional value of conventional and modified DDGS for poultry* in *Feeding and Nutrition Conference*, P.M.-S. Poultry, Editor 2006: Indianapolis.
5. AAFCO, *Association of American Feed Control Officials*, 2007.
6. Wisner, R., *estimated U.S Dried Distillers Grains with Solubles (DDGS) Production and Use*, 2013, agricultural marketing resource center
7. Stevens, C.E., *African Corn-Fields - a Review.* Antiquity, 1958. **32**(125): p. 25-29.
8. http://pedagog.hubpages.com/hub/Why-Pop-corns-Pop#).
9. *Renewable Fuel Association*, 2009.
10. Liu, K., *Chemical composition of distillers grains, a review*. J Agric Food Chem, 2011. **59**(5): p. 1508-26.
11. Watkins, C., *Two fuels from one kernel*. Vol. Inform 18: 714-717. 2007.
12. Rausch, K.D. and R.L. Belyea, *The future of coproducts from corn processing.* Applied Biochemistry and Biotechnology, 2006. **128**(1): p. 47-86.
13. Stein, H.H. and G.C. Shurson, *BOARD-INVITED REVIEW: The use and application of distillers dried grains with solubles in swine diets.* Journal of Animal Science, 2009. **87**(4): p. 1292-1303.
14. Boruf, C.S., J. Miller., *Solvent extraction of corn oil from distillers grains* Journal of the American Oil Chemists Society, 1937. **14: 321-313**.
15. Keshun Liu, K.A.R., *Distillers Grains: Production, Properties, and Utilization*2011, CRC Press.
16. Balat, M. and H. Balat, *Recent trends in global production and utilization of bio-ethanol fuel.* Applied Energy, 2009. **86**(11): p. 2273-2282.
17. Outlook, E.I., *Ethanol Industry Outlook*, 2010, Renewable Fuels Association: Washington, DC.
18. Belyea, R.L., K.D. Rausch, and M.E. Tumbleson, *Composition of corn and distillers dried grains with solubles from dry grind ethanol processing.* Bioresource Technology, 2004. **94**(3): p. 293-298.
19. Vijay Singh, D.J., Kalpana Naidu, Kent Rausch, *Comparison of Modified Dry-Grind Corn Processes for Fermentation Characteristics and DDGS Composition.* Cereal Chemistry, 2005. **82**: p. 187-190.
20. Kim, Y., et al., *Composition of corn dry-grind ethanol by-products: DDGS, wet cake, and thin stillage.* Bioresour Technol, 2008. **99**(12): p. 5165-76.

21. Leguizamon, C., et al., *Plant Sterol and Policosanol Characterization of Hexane Extracts from Grain Sorghum, Corn and their DDGS.* Journal of the American Oil Chemists Society, 2009. **86**(7): p. 707-716.
22. Majoni, S. and T. Wang, *Characterization of Oil Precipitate and Oil Extracted from Condensed Corn Distillers Solubles.* Journal of the American Oil Chemists Society, 2010. **87**(2): p. 205-213.
23. Moreau, *Changes in lipid composition during dry grind ethanol processing of corn.* Journal of the American Oil Chemists Society, 2010.
24. Jenkins, T.C., *Lipid metabolism in the rumen.* J Dairy Sci, 1993. **76**(12): p. 3851-63.
25. Roberson, K.D., J.K.Kalbfleisch, W.Pan, and R.A. Charbeneau, *Effects of Corn Distiller's Dried Grains with Solubles at Various Levels on Performance of Laying Hens and Egg Yolk Color* Int. J. Poult. Sci, 2005. **4: 44-51**.
26. NRC, *Feeding Value of Ethanol Production By-products*, 1981, National Research Council: Washinton D.C.
27. Sauvant, D.a.G.T., *Corn distillers*. Tables of composition and nutritional value of feed materials. 2004, Netherlands: Wageningen Academic Publishers.
28. Batal, A.B. and N.M. Dale, *True metabolizable energy and amino acid digestibility of distillers dried grains with solubles.* Journal of Applied Poultry Research, 2006. **15**(1): p. 89-93.
29. Robinson, P.H., K. Karges, and M.L. Gibson, *Nutritional evaluation of four co-product feedstuffs from the motor fuel ethanol distillation industry in the Midwestern USA.* Animal Feed Science and Technology, 2008. **146**(3-4): p. 345-352.
30. Whitney, M.H. and G.C. Shurson, *Growth performance of nursery pigs fed diets containing increasing levels of corn distiller's dried grains with solubles originating from a modern Midwestern ethanol plant.* J Anim Sci, 2004. **82**(1): p. 122-8.
31. Jiang, W., L. Zhang, and A. Shan, *The effect of vitamin E on laying performance and egg quality in laying hens fed corn dried distillers grains with solubles.* Poult Sci, 2013. **92**(11): p. 2956-64.
32. Masa'deh, M.K., *Dried Distillers Grain with Solubles in Laying Hen and Pullet Rations*, 2011, University of Nebraska-Lincoln
33. Tran, H., et al., *Effect of corn distillers dried grains with solubles on growth performance and health status indicators in weanling pigs.* J Anim Sci, 2012. **90**(3): p. 790-801.
34. Abbott, J., J.O'Palka, *Dried ditillers' grains with solubles: Particle size effects on volume and acceptability of baked products.* Journal of Food Science, 1991. **56(5): 1323-1326**.
35. Ahmed, J., *Utlization of corn distillers' grains in chipathi*, in *South Dakota State University*1995.
36. Arra, S., P.G.Krishnan, *Fortifying chapathis, an Asian whole wheat unleavened bread, using corn distillers dried grains, Institute of Food Technology 2009*2009: Chicago, IL.

37. Bookwalter, G.N., K . Warner, J.S.Wall, and Y.V.Wu, *Corn distillers grain and other by-products of alcohol production in blended foods. Sensory, stability, and processing studies.* . Cereal Chemistry, 1984. **61(6): 509-513**.
38. Brochetti, D., M.P.Penfield, and M.F.Heim-Edelman, *Yeast bread containinb distillers dried grain dough development and bread quality.* Journal of Food quality, 1991. **14(4):331-344**.
39. Jessica A. Herr Saunders, R.D., *Potential bleaching techniques for use in Distillers Grains*, in *ASABE Meeting*2007: Minneaplis, Minnesota.
40. Kim, C.H., J.A. Maga, and J.T. Martin, *Properties of Extruded Blends of Wheat Dried Distiller Grain Flour with Other Flours.* International Journal of Food Science and Technology, 1989. **24**(4): p. 373-384.
41. Nrochetti, D., and M.P.Penfield, *Sensory characteristics of bakery products containing distillers' dried grain from corn, barley and rye.* Journal of Food Quality, 1989. **12: 413-426**.
42. Brochetti, D. and M.P. Penfield, *Sensory Characteristics of Bakery Products Containing Distillers Dried Grain from Corn, Barley, and Rye.* Journal of Food Quality, 1989. **12**(6): p. 413-426.
43. ADA, *Dietary Fiber*, 2002, American Dietetic Association.
44. Gutkoski, L.C., et al., *Development of oat based cereal bars with high dietary fiber content.* Ciencia E Tecnologia De Alimentos, 2007. **27**(2): p. 355-363.
45. Palacio, J.C. and J.L. Rombeau, *Dietary fiber: a brief review and potential application to enteral nutrition.* Nutr Clin Pract, 1990. **5**(3): p. 99-106.
46. Quaade, F., E. Vrist, and A. Astrup, *[Dietary fiber added to a very-low caloric diet reduces hunger and alleviates constipation]*. Ugeskr Laeger, 1990. **152**(2): p. 95-8.
47. Chemists, A.A.o.C., *the defination of dietary fiber* 20001.
48. Arjmandi, B.H., et al., *Soluble Dietary Fiber and Cholesterol Influence Invivo Hepatic and Intestinal Cholesterol-Biosynthesis in Rats.* Journal of Nutrition, 1992. **122**(7): p. 1559-1565.
49. Haskell, W.L., et al., *Role of Water-Soluble Dietary Fiber in the Management of Elevated Plasma-Cholesterol in Healthy-Subjects.* American Journal of Cardiology, 1992. **69**(5): p. 433-439.
50. Masse, J., *Water-Soluble Dietary Fiber and Low-Density-Lipoprotein Cholesterol.* American Journal of Cardiology, 1992. **70**(7): p. 840-840.
51. Anderson, J.W., et al., *Health benefits of dietary fiber.* Nutr Rev, 2009. **67**(4): p. 188-205.
52. Chawla, R. and G.R. Patil, *Soluble Dietary Fiber.* Comprehensive Reviews in Food Science and Food Safety, 2010. **9**(2): p. 178-196.
53. Cho, S.S., et al., *Consumption of cereal fiber, mixtures of whole grains and bran, and whole grains and risk reduction in type 2 diabetes, obesity, and cardiovascular disease.* Am J Clin Nutr, 2013. **98**(2): p. 594-619.
54. Nishimura, N., Y. Taniguchi, and S. Kiriyama, *Plasma cholesterol-lowering effect on rats of dietary fiber extracted from immature plants.* Biosci Biotechnol Biochem, 2000. **64**(12): p. 2543-51.

55. Mumford, S.L., et al., *Effect of dietary fiber intake on lipoprotein cholesterol levels independent of estradiol in healthy premenopausal women.* Am J Epidemiol, 2011. **173**(2): p. 145-56.
56. Sarria, B., et al., *Regular consumption of a soluble cocoa product rich in dietary fibre increases hdl-cholesterol without inducing anthropometric changes in healthy and hypercholesterolemic subjects.* Annals of Nutrition and Metabolism, 2011. **58**: p. 235-235.
57. Juhel, C., et al., *Cholesterol-lowering effect of non-viscous soluble dietary fiber NUTRIOSE (R) 6 in moderately hypercholesterolemic hamsters.* Indian Journal of Experimental Biology, 2011. **49**(3): p. 219-228.
58. Samuel, P., et al., *Comparison of body mass index (BMI) and serum cholesterol concentration by quartiles of dietary fiber consumption in adults: the Third National Health and Nutrition Survey (NHANES III, 1988-1994).* Obesity Research, 2000. **8**: p. 106s-106s.
59. Anderson, J.W., et al., *Health benefits of dietary fiber.* Nutrition Reviews, 2009. **67**(4): p. 188-205.
60. Whelton, S.P., et al., *Effect of dietary fiber intake on blood pressure: a meta-analysis of randomized, controlled clinical trials.* Journal of Hypertension, 2005. **23**(3): p. 475-481.
61. US Department of Agriculture, N.A.L.a.N.A.o.S., *Dietary, Functional and Total fibre*, C. Dietary Reference Intakes for Energy, fibre, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients), Editor 2005, Institute of Medicine, Food and Nutrition Board.
62. Yang, J., A. Keshavarzian, and D.J. Rose, *Impact of dietary fiber fermentation from cereal grains on metabolite production by the fecal microbiota from normal weight and obese individuals.* J Med Food, 2013. **16**(9): p. 862-7.
63. Bourquin, L.D., E.C. Titgemeyer, and G.C. Fahey, Jr., *Vegetable fiber fermentation by human fecal bacteria: cell wall polysaccharide disappearance and short-chain fatty acid production during in vitro fermentation and water-holding capacity of unfermented residues.* J Nutr, 1993. **123**(5): p. 860-9.
64. Anderson, J.W. and S.R. Bridges, *Short-Chain Fatty-Acid Fermentation Products of Plant Fiber Affect Glucose-Metabolism of Isolated Rat Hepatocytes.* Proceedings of the Society for Experimental Biology and Medicine, 1984. **177**(2): p. 372-376.
65. Bourquin, L.D., et al., *Fermentation of Dietary Fiber by Human Colonic Bacteria - Disappearance of, Short-Chain Fatty-Acid Production from, and Potential Water-Holding Capacity of, Various Substrates.* Scandinavian Journal of Gastroenterology, 1993. **28**(3): p. 249-255.
66. Ramakrishna, B.S., *Role of the gut microbiota in human nutrition and metabolism.* J Gastroenterol Hepatol, 2013. **28 Suppl 4**: p. 9-17.
67. Cook, S.I. and J.H. Sellin, *Review article: short chain fatty acids in health and disease.* Aliment Pharmacol Ther, 1998. **12**(6): p. 499-507.
68. Kato, T., H. Hayashi, and Y. Sugiyama, *Short- and medium-chain fatty acids enhance the cell surface expression and transport capacity of the bile salt export*

pump (BSEP/ABCB11). Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids, 2010. **1801**(9): p. 1005-1012.
69. Backhed, F., et al., *Host-bacterial mutualism in the human intestine.* Science, 2005. **307**(5717): p. 1915-1920.
70. Hamer, H.M., et al., *Review article: the role of butyrate on colonic function.* Alimentary Pharmacology & Therapeutics, 2008. **27**(2): p. 104-119.
71. Wong, J.M.W., et al., *Colonic health: Fermentation and short chain fatty acids.* Journal of Clinical Gastroenterology, 2006. **40**(3): p. 235-243.
72. Abdel-Aal, E.M., et al., *Dietary Sources of Lutein and Zeaxanthin Carotenoids and Their Role in Eye Health.* Nutrients, 2013. **5**(4): p. 1169-1185.
73. Moreau, R.A., D.B. Johnston, and K.B. Hicks, *A comparison of the levels of lutein and zeaxanthin in corn germ oil, corn fiber oil and corn kernel oil.* Journal of the American Oil Chemists Society, 2007. **84**(11): p. 1039-1044.
74. Deming, D.M. and J.W. Erdman, *Mammalian carotenoid absorption and metabolism.* Pure and Applied Chemistry, 1999. **71**(12): p. 2213-2223.
75. Castenmiller, J.J.M., et al., *The food matrix of spinach is a limiting factor in determining the bioavailability of beta-carotene and to a lesser extent of lutein in humans.* Journal of Nutrition, 1999. **129**(2): p. 349-355.
76. Williams, A.W., T.W.M. Boileau, and J.W. Erdman, *Factors influencing the uptake and absorption of carotenoids.* Proceedings of the Society for Experimental Biology and Medicine, 1998. **218**(2): p. 106-108.
77. Parker, R.S., *Absorption, metabolism, and transport of carotenoids.* FASEB J, 1996. **10**(5): p. 542-51.
78. Erdman, J.W., Jr., T.L. Bierer, and E.T. Gugger, *Absorption and transport of carotenoids.* Ann N Y Acad Sci, 1993. **691**: p. 76-85.
79. Parker, R.S., *Carotenoids .4. Absorption, metabolism, and transport of carotenoids.* Faseb Journal, 1996. **10**(5): p. 542-551.
80. Riedl, J., et al., *Some dietary fibers reduce the absorption of carotenoids in women.* Journal of Nutrition, 1999. **129**(12): p. 2170-2176.
81. Hoffmann, J., et al., *Dietary fiber reduces the antioxidative effect of a carotenoid and alpha-tocopherol mixture on LDL oxidation ex vivo in humans.* European Journal of Nutrition, 1999. **38**(6): p. 278-285.
82. Roodenburg, A.J., et al., *Amount of fat in the diet affects bioavailability of lutein esters but not of alpha-carotene, beta-carotene, and vitamin E in humans.* Am J Clin Nutr, 2000. **71**(5): p. 1187-93.
83. Khachik, F., et al., *The effect of lutein and zeaxanthin supplementation on metabolites of these carotenoids in the serum of persons aged 60 or older.* Invest Ophthalmol Vis Sci, 2006. **47**(12): p. 5234-42.
84. Lakshminarayana, R., et al., *Possible degradation/biotransformation of lutein in vitro and in vivo: isolation and structural elucidation of lutein metabolites by HPLC and LC-MS (atmospheric pressure chemical ionization).* Free Radic Biol Med, 2008. **45**(7): p. 982-93.
85. Yonekura, L., et al., *Keto-carotenoids are the major metabolites of dietary lutein and fucoxanthin in mouse tissues.* J Nutr, 2010. **140**(10): p. 1824-31.

86. Chew, B.P., M.W. Wong, and T.S. Wong, *Effects of lutein from marigold extract on immunity and growth of mammary tumors in mice.* Anticancer Research, 1996. **16**(6B): p. 3689-3694.
87. Snodderly, D.M., *Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins.* Am J Clin Nutr, 1995. **62**(6 Suppl): p. 1448S-1461S.
88. Kritchevsky, S.B., *beta-carotene, carotenoids and the prevention of coronary heart disease.* Journal of Nutrition, 1999. **129**(1): p. 5-8.
89. Ascherio, A., et al., *Relation of consumption of vitamin E, vitamin C, and carotenoids to risk for stroke among men in the United States.* Annals of Internal Medicine, 1999. **130**(12): p. 963-970.
90. Holick, C.N., et al., *Dietary carotenoids, serum beta-carotene, and retinol and risk of lung cancer in the alpha-tocopherol, beta-carotene cohort study.* American Journal of Epidemiology, 2002. **156**(6): p. 536-547.
91. Connor, S.L., et al., *Diets lower in folic acid and carotenoids are associated with the coronary disease epidemic in Central and Eastern Europe.* Journal of the American Dietetic Association, 2004. **104**(12): p. 1793-1799.
92. Martin, K.R., D. Wu, and M. Meydani, *The effect of carotenoids on the expression of cell surface adhesion molecules and binding of monocytes to human aortic endothelial cells.* Atherosclerosis, 2000. **150**(2): p. 265-274.
93. Iribarren, C., et al., *Association of serum vitamin levels, LDL susceptibility to oxidation, and autoantibodies against MDA-LDL with carotid atherosclerosis - A case-control study.* Arteriosclerosis Thrombosis and Vascular Biology, 1997. **17**(6): p. 1171-1177.
94. Wang, X. and S. Hu, *[A review of research actuality on age-related macular degeneration]*. Yan Ke Xue Bao, 2001. **17**(4): p. 245-51.
95. Dasari, B., et al., *Cholesterol-enriched diet causes age-related macular degeneration-like pathology in rabbit retina.* BMC Ophthalmol, 2011. **11**: p. 22.
96. de Jong, P.T., *Age-related macular degeneration.* N Engl J Med, 2006. **355**(14): p. 1474-85.
97. Krinsky, N.I., J.T. Landrum, and R.A. Bone, *Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye.* Annu Rev Nutr, 2003. **23**: p. 171-201.
98. Bone, R.A., et al., *Macular pigment in donor eyes with and without AMD: a case-control study.* Invest Ophthalmol Vis Sci, 2001. **42**(1): p. 235-40.
99. Zhang, X., et al., *Macular pigment and visual acuity in Stargardt macular dystrophy.* Graefes Arch Clin Exp Ophthalmol, 2002. **240**(10): p. 802-9.
100. Seddon, J.M. and C.A. Chen, *The epidemiology of age-related macular degeneration.* Int Ophthalmol Clin, 2004. **44**(4): p. 17-39.
101. Curran Celentano, J., J.D. Burke, and B.R. Hammond, Jr., *In vivo assessment of retinal carotenoids: macular pigment detection techniques and their impact on monitoring pigment status.* J Nutr, 2002. **132**(3): p. 535S-539S.

102. Seddon, J.M., et al., *Dietary Carotenoids, Vitamin-a, Vitamin-C, and Vitamin-E, and Advanced Age-Related Macular Degeneration.* Jama-Journal of the American Medical Association, 1994. **272**(18): p. 1413-1420.
103. Flood, V., et al., *Lutein and zeaxanthin dietary intake and age related macular degeneration.* Br J Ophthalmol, 2006. **90**(7): p. 927-8.
104. Rai, A.K., P. Debetto, and F.D. Sala, *Molecular regulation of cholesterol metabolism: HDL-based intervention through drugs and diet.* Indian Journal of Experimental Biology, 2013. **51**(11): p. 885-94.
105. Bertolotti, M., D.K. Spady, and J.M. Dietschy, *Regulation of hepatic cholesterol metabolism in the rat in vivo: effect of a synthetic fat-free diet on sterol synthesis and low-density lipoprotein transport.* Biochim Biophys Acta, 1995. **1255**(3): p. 293-300.
106. Liu, G.F., et al., *Expression of HMGR and corresponding cholesterol content in tissues of two pig breeds.* Animal, 2009. **3**(10): p. 1436-41.
107. Clarke, G.A., et al., *Cholesterol biosynthesis inhibitors acting distally to 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) augment secretion of all biliary lipids in lithogenic mice but high doses uncouple phosphatidylcholine (PC) from cholesterol (Ch) secretion.* Gastroenterology, 2001. **120**(5): p. A551-A551.
108. Angelin, B., *1994 Mack-Forster Award Lecture. Review. Studies on the regulation of hepatic cholesterol metabolism in humans.* Eur J Clin Invest, 1995. **25**(4): p. 215-24.
109. Ferezou, J., et al., *Daily elimination of fecal neutral sterols by humans.* Digestion, 1978. **18**(3-4): p. 201-12.
110. Reddy, B.S. and E.L. Wynder, *Large-bowel carcinogenesis: fecal constituents of populations with diverse incidence rates of colon cancer.* J Natl Cancer Inst, 1973. **50**(6): p. 1437-42.
111. B. S. Drasar, J.S.C., *the relation between diet and gut microflora in man.* Proc Nutr Soc, 1973. **32**: p. 49.
112. Gleeson, A., et al., *The relationship between cholesterol absorption and intestinal cholesterol synthesis in the diabetic rat model.* Int J Exp Diabetes Res, 2000. **1**(3): p. 203-10.
113. Wilson, M.D. and L.L. Rudel, *Review of cholesterol absorption with emphasis on dietary and biliary cholesterol.* J Lipid Res, 1994. **35**(6): p. 943-55.
114. Knopp, R.H., et al., *Long-term blood cholesterol-lowering effects of a dietary fiber supplement.* Am J Prev Med, 1999. **17**(1): p. 18-23.
115. Brown, A., D. Wiggins, and G.F. Gibbons, *Manipulation of cholesterol and cholesteryl ester synthesis has multiple effects on the metabolism of apolipoprotein B and the secretion of very-low-density lipoprotein by primary hepatocyte cultures.* Biochim Biophys Acta, 1999. **1440**(2-3): p. 253-65.
116. Garcia-Calvo, M., et al., *The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1).* Proc Natl Acad Sci U S A, 2005. **102**(23): p. 8132-7.
117. Yu, L., et al., *Cholesterol-regulated translocation of NPC1L1 to the cell surface facilitates free cholesterol uptake.* J Biol Chem, 2006. **281**(10): p. 6616-24.

118. Cohen, J.C., et al., *Multiple rare variants in NPC1L1 associated with reduced sterol absorption and plasma low-density lipoprotein levels.* Proc Natl Acad Sci U S A, 2006. **103**(6): p. 1810-1815.
119. J. Koolman, K.H.R., *color atlas of biochemistry*2005: Thieme Stuttgart. New York.
120. Lane, D.M., *Third report of the National Cholesterol Education Program (NCEP. III): A switch from dietary modification to risk factor assessment.* Curr Opin Investig Drugs, 2001. **2**(9): p. 1235-6.
121. Murakami, S., Y. Kondo, and T. Nagate, *Effects of long-term treatment with taurine in mice fed a high-fat diet - Improvement in cholesterol metabolism and vascular lipid accumulation by taurine.* Taurine 4: Taurine and Excitable Tissues, 2000. **483**: p. 177-186.
122. Colquhoun, D.T., *Food for prevention of coronary heart disease: Beyond the low fat, low cholesterol diet.* Asia Pacific Journal of Clinical Nutrition, 2000. **9**: p. S86-S90.
123. USDA, *Cholesterol intake recommendation*, 2010.
124. Bazzano, L.A., *Effects of soluble dietary fiber on low-density lipoprotein cholesterol and coronary heart disease risk.* Curr Atheroscler Rep, 2008. **10**(6): p. 473-7.
125. Cho, I.J., C. Lee, and T.Y. Ha, *Hypolipidemic effect of soluble fiber isolated from seeds of Cassia tora Linn. in rats fed a high-cholesterol diet.* J Agric Food Chem, 2007. **55**(4): p. 1592-6.
126. Ramos, S., et al., *Hypolipidemic effect in cholesterol-fed rats of a soluble fiber-rich product obtained from cocoa husks.* J Agric Food Chem, 2008. **56**(16): p. 6985-93.
127. Shen, H., et al., *Dietary soluble fiber lowers plasma LDL cholesterol concentrations by altering lipoprotein metabolism in female guinea pigs.* J Nutr, 1998. **128**(9): p. 1434-41.
128. Gunness, P. and M.J. Gidley, *Mechanisms underlying the cholesterol-lowering properties of soluble dietary fibre polysaccharides.* Food Funct, 2010. **1**(2): p. 149-55.
129. Tiwari, U. and E. Cummins, *Meta-analysis of the effect of beta-glucan intake on blood cholesterol and glucose levels.* Nutrition, 2011. **27**(10): p. 1008-16.
130. Florakis, D., et al., *Sustained reduction in circulating cholesterol in adult hypopituitary patients given low dose titrated growth hormone replacement therapy: a two year study*. Clin Endocrinol (Oxf), 2000. **53**(4): p. 453-9.
131. Brunzell, J.D., et al., *Lipoprotein management in patients with cardiometabolic risk - Consensus conference report from the American diabetes association and the American college of cardiology foundation.* Journal of the American College of Cardiology, 2008. **51**(15): p. 1512-1524.
132. Kizer, J.R., et al., *Relation of Different Measures of Low-Density Lipoprotein Cholesterol to Risk of Coronary Artery Disease and Death in a Meta-Regression Analysis of Large-Scale Trials of Statin Therapy.* American Journal of Cardiology, 2010. **105**(9): p. 1289-1296.

133. Bocharova, L.V., *[Bile acid metabolism (review of the literature)]*. Sov Med, 1985(5): p. 84-7.
134. McCashland, T.M., et al., *Bile acid metabolism and biliary secretion in patients receiving orthotopic liver transplants: differing effects of cyclosporine and FK 506.* Hepatology, 1994. **19**(6): p. 1381-9.
135. http://en.wikipedia.org/wiki/Bile_acid.
136. Vlahcevic, Z.R., *Regulation of cholesterol 7 alpha-hydroxylase by different effectors.* Ital J Gastroenterol, 1996. **28**(6): p. 337-9.
137. Twisk, J., et al., *Heterogeneous expression of cholesterol 7 alpha-hydroxylase and sterol 27-hydroxylase genes in the rat liver lobulus.* J Clin Invest, 1995. **95**(3): p. 1235-43.
138. Repa, J.J. and D.J. Mangelsdorf, *Nuclear receptor regulation of cholesterol and bile acid metabolism.* Curr Opin Biotechnol, 1999. **10**(6): p. 557-63.
139. Schwarz, M., et al., *Marked reduction in bile acid synthesis in cholesterol 7alpha-hydroxylase-deficient mice does not lead to diminished tissue cholesterol turnover or to hypercholesterolemia.* J Lipid Res, 1998. **39**(9): p. 1833-43.
140. Horton, J.D., J.A. Cuthbert, and D.K. Spady, *Regulation of hepatic 7 alpha-hydroxylase expression and response to dietary cholesterol in the rat and hamster.* J Biol Chem, 1995. **270**(10): p. 5381-7.
141. Marie Borum, K.S., Hans Fromm, Saleem Jahangeer, Marianne Floor, *Fecal bile acid excretion and composition in response to changes in dierary wheat bran, fat and calcium in the rat.* Lipid, 1992. **27**(No. 12): p. 999-1004.
142. Turjman, N. and C. Jacob, *Diet, nutrition intake, and metabolism in populations at high and low risk for colon cancer. Amberlite XAD-2 in the determination of human fecal bile acids.* Am J Clin Nutr, 1984. **40**(4 Suppl): p. 957-60.
143. Bell, E.W., et al., *Effects of dietary fiber from wheat, corn, and soy hull bran on excretion of fecal bile acids in humans.* Am J Clin Nutr, 1981. **34**(6): p. 1071-6.
144. Reddy, B.S., et al., *Metabolic epidemiology of colon cancer: effect of dietary fiber on fecal mutagens and bile acids in healthy subjects.* Cancer Res, 1987. **47**(2): p. 644-8.
145. Vahouny, G.V., et al., *Dietary fiber supplementation and fecal bile acids, neutral steroids and divalent cations in rats.* J Nutr, 1987. **117**(12): p. 2009-15.
146. Anderson, J.W., et al., *Oat-bran cereal lowers serum total and LDL cholesterol in hypercholesterolemic men.* Am J Clin Nutr, 1990. **52**(3): p. 495-9.
147. Alberts, D.S., et al., *Randomized, double-blinded, placebo-controlled study of effect of wheat bran fiber and calcium on fecal bile acids in patients with resected adenomatous colon polyps.* J Natl Cancer Inst, 1996. **88**(2): p. 81-92.
148. Kishimoto, Y., S. Wakabayashi, and H. Takeda, *Hypocholesterolemic effect of dietary fiber: relation to intestinal fermentation and bile acid excretion.* J Nutr Sci Vitaminol (Tokyo), 1995. **41**(1): p. 151-61.
149. Kahlon, T.S. and F.I. Chow, *In vitro binding of bile acids by rice bran, oat bran, wheat bran, and corn bran.* Cereal Chemistry, 2000. **77**(4): p. 518-521.
150. Dantas, A.P., F. Jimenez-Altayo, and E. Vila, *Vascular aging: facts and factors.* Front Physiol, 2012. **3**: p. 325.

151. Titov, V.N., *[Common features of atherosclerosis and inflammation: specificity of atherosclerosis as an inflammatory process (a hypothesis)]*. Klin Lab Diagn, 2000(4): p. 3-10.
152. Finegold, J.A., P. Asaria, and D.P. Francis, *Mortality from ischaemic heart disease by country, region, and age: Statistics from World Health Organisation and United Nations.* International Journal of Cardiology, 2013. **168**(2): p. 934-945.
153. Ignarro, L.J., M.L. Balestrieri, and C. Napoli, *Nutrition, physical activity, and cardiovascular disease: an update.* Cardiovasc Res, 2007. **73**(2): p. 326-40.
154. Eastham, R.D. and J. Jancar, *Plasma-Cholesterol, Coronary Heart-Disease, and Cancer*. British Medical Journal, 1989. **298**(6681): p. 1176-1176.
155. Goren, R. and J.B. Hickie, *Cholesterol Esterification in Plasma (Lcat), in Coronary Heart-Disease.* Australian and New Zealand Journal of Medicine, 1975. **5**(1): p. 86-86.
156. Ezhov, M.V., et al., *Relationship between high lipoprotein (a) level and severe coronary atherosclerosis in patients with ischemic heart disease with normal plasma concentration of cholesterol and triglycerides.* Kardiologiya, 1997. **37**(11): p. 18-22.
157. Gordon, D.J., *Plasma High-Density Lipoprotein Cholesterol and Coronary Heart-Disease in Hypercholesterolemic Men.* Circulation, 1985. **72**(4): p. 185-185.
158. Dwyer, J.H., et al., *Oxygenated carotenoid lutein and progression of early atherosclerosis - The Los Angeles Atherosclerosis Study.* Circulation, 2001. **103**(24): p. 2922-2927.
159. Paradies, G., et al., *The effect of reactive oxygen species generated from the mitochondrial electron transport chain on the cytochrome c oxidase activity and on the cardiolipin content in bovine heart submitochondrial particles.* Febs Letters, 2000. **466**(2-3): p. 323-326.
160. Maziere, C., et al., *Oxidized LDL induces an oxidative stress and activates the tumor suppressor p53 in MRC5 human fibroblasts.* Biochemical and Biophysical Research Communications, 2000. **276**(2): p. 718-723.
161. Song, R., *Lipid peroxidation in corn dried distillers grains with solybles and effecrs of feefing a highly oxidized DDGS source to swine*, in *Animal science*2013, UMN.
162. Palmquist, D.L. and T.C. Jenkins, *Challenges with fats and fatty acid methods.* Journal of Animal Science, 2003. **81**(12): p. 3250-3254.
163. Lee, H.S., D.W. Shoeman, and A.S. Csallany, *Urinary Response to Invivo Lipid-Peroxidation Induced by Vitamin-E-Deficiency.* Lipids, 1992. **27**(2): p. 124-128.
164. Folch, J., M. Lees, and G.H. Sloane Stanley, *A simple method for the isolation and purification of total lipides from animal tissues.* J Biol Chem, 1957. **226**(1): p. 497-509.
165. Gallaher, D.D., et al., *Viscosity and fermentability as attributes of dietary fiber responsible for the hypocholesterolemic effect in hamsters.* J Nutr, 1993. **123**(2): p. 244-52.

166. Locket, P.L. and D.D. Gallaher, *An improved procedure for bile acid extraction and purification and tissue distribution in the rat.* Lipids, 1989. **24**(3): p. 221-3.
167. Sheltawy, M.J. and M.S. Losowsky, *Determination of faecal bile acids by an enzymic method.* Clin Chim Acta, 1975. **64**(2): p. 127-32.
168. Lei Li, J.Q., Shi-an Yin, Guangwen Tang, *Effects of mobile phase ratios on the separation of plant carotenoids by HPLC with a C30 column.* Chromatographia, 2007. **65**(1-2): p. 91-94.
169. Crupi, P., R.A. Milella, and D. Antonacci, *Simultaneous HPLC-DAD-MS (ESI+) determination of structural and geometrical isomers of carotenoids in mature grapes.* J Mass Spectrom, 2010. **45**(9): p. 971-80.
170. Rivera, S., F. Vilaro, and R. Canela, *Determination of carotenoids by liquid chromatography/mass spectrometry: effect of several dopants.* Anal Bioanal Chem, 2011. **400**(5): p. 1339-46.
171. Schlatterer, J. and D.E. Breithaupt, *Xanthophylls in commercial egg yolks: quantification and identification by HPLC and LC-(APCI)MS using a C30 phase.* J Agric Food Chem, 2006. **54**(6): p. 2267-73.
172. Whitney, M.H., et al., *Growth performance and carcass characteristics of grower-finisher pigs fed high-quality corn distillers dried grain with solubles originating from a modern Midwestern ethanol plant.* J Anim Sci, 2006. **84**(12): p. 3356-3363.
173. Garcia, J., et al., *Effect of fiber source on cecal fermentation and nitrogen recycled through cecotrophy in rabbits.* J Anim Sci, 2000. **78**(3): p. 638-46.
174. Hara, H., et al., *Fermentation products of sugar-beet fiber by cecal bacteria lower plasma cholesterol concentration in rats.* J Nutr, 1998. **128**(4): p. 688-93.
175. Miyasaka, H., H. Sanada, and Y. Ayano, *Relationship between Colonic Products from Corn Bran Hemicellulose and the Lipid-Metabolism in Rats Fed on Diets Supplemented with Orotic-Acid.* Bioscience Biotechnology and Biochemistry, 1992. **56**(1): p. 157-158.
176. Fleming, S.E., D. Marthinsen, and H. Kuhnlein, *Colonic function and fermentation in men consuming high fiber diets.* J Nutr, 1983. **113**(12): p. 2535-44.
177. Mongeau, R., S.H. Yiu, and R. Brassard, *Chemical and fluorescence microscopic analysis of fiber degradation of oat, Hard Red Spring wheat, and corn bran in rats.* Journal of Agricultural and Food Chemistry, 1991. **39**(11): p. 1966-1971.
178. Dewanto, V., X.Z. Wu, and R.H. Liu, *Processed sweet corn has higher antioxidant activity.* Journal of Agricultural and Food Chemistry, 2002. **50**(17): p. 4959-4964.
179. Oh, S.Y. and S. Chang, *Antioxidant effects of squalene on corn oil fed mice.* Faseb Journal, 2002. **16**(5): p. A1010-A1011.
180. Kanski, J., et al., *Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems in vitro: structure-activity studies.* J Nutr Biochem, 2002. **13**(5): p. 273-281.

181. Srinivasan, M., A.R. Sudheer, and V.P. Menon, *Ferulic Acid: therapeutic potential through its antioxidant property.* J Clin Biochem Nutr, 2007. **40**(2): p. 92-100.
182. Zhao, Z., Y. Egashira, and H. Sanada, *Phenolic antioxidants richly contained in corn bran are slightly bioavailable in rats.* J Agric Food Chem, 2005. **53**(12): p. 5030-5.
183. Ahmed, S.S., M.N. Lott, and D.M. Marcus, *The macular xanthophylls.* Surv Ophthalmol, 2005. **50**(2): p. 183-93.
184. Moukarzel, A.A., R.A. Bejjani, and F.N. Fares, *Xanthophylls and eye health of infants and adults.* J Med Liban, 2009. **57**(4): p. 261-7.
185. Niyogi, K.K., O. Bjorkman, and A.R. Grossman, *The roles of specific xanthophylls in photoprotection.* Proc Natl Acad Sci U S A, 1997. **94**(25): p. 14162-7.
186. Krinsky, N.I., *Possible biologic mechanisms for a protective role of xanthophylls.* J Nutr, 2002. **132**(3): p. 540S-542S.
187. Krinsky, N.I., *The antioxidant and biological properties of the carotenoids.* Ann N Y Acad Sci, 1998. **854**: p. 443-7.
188. Torbergsen, A.C. and A.R. Collins, *Recovery of human lymphocytes from oxidative DNA damage; the apparent enhancement of DNA repair by carotenoids is probably simply an antioxidant effect.* European Journal of Nutrition, 2000. **39**(2): p. 80-85.
189. Collins, A.R., et al., *Serum carotenoids and oxidative DNA damage in human lymphocytes.* Carcinogenesis, 1998. **19**(12): p. 2159-62.
190. Weber, T.E. and B.J. Kerr, *Effect of dietary distillers dried grains with solubles on indicators of oxidative stress and immune function in growing pigs.* Livestock Science, 2011. **142**(1-3): p. 85-91.
191. Reddy, B.S., Y. Maeura, and M. Wayman, *Effect of dietary corn bran and autohydrolyzed lignin on 3,2'-dimethyl-4-aminobiphenyl-induced intestinal carcinogenesis in male F344 rats.* J Natl Cancer Inst, 1983. **71**(2): p. 419-23.
192. Lopez, H.W., et al., *Effects of soluble corn bran arabinoxylans on cecal digestion, lipid metabolism, and mineral balance (Ca, Mg) in rats.* J Nutr Biochem, 1999. **10**(9): p. 500-9.
193. Rodriguez, R., et al., *Dietary fibre from vegetable products as source of functional ingredients.* Trends in Food Science & Technology, 2006. **17**(1): p. 3-15.
194. Gunness, P. and M.J. Gidley, *Mechanisms underlying the cholesterol-lowering properties of soluble dietary fibre polysaccharides.* Food & Function, 2010. **1**(2): p. 149-155.
195. Lia, A., et al., *Oat beta-glucan increases bile acid excretion and a fiber-rich barley fraction increases cholesterol excretion in ileostomy subjects.* Am J Clin Nutr, 1995. **62**(6): p. 1245-51.
196. Stark, A., et al., *Changes in Intestinal Tunica Muscularis Following Dietary Fiber Feeding in Rats - a Morphometric Study Using Image-Analysis.* Dig Dis Sci, 1995. **40**(5): p. 960-966.

197. Erkkila, A.T. and A.H. Lichtenstein, *Fiber and cardiovascular disease risk: how strong is the evidence?* J Cardiovasc Nurs, 2006. **21**(1): p. 3-8.
198. Juntunen, K.S., et al., *High-fiber rye bread and insulin secretion and sensitivity in healthy postmenopausal women.* Am J Clin Nutr, 2003. **77**(2): p. 385-91.
199. Kim, H.J. and P.J. White, *In vitro bile-acid binding and fermentation of high, medium, and low molecular weight beta-glucan.* J Agric Food Chem, 2010. **58**(1): p. 628-34.
200. Rodriguez-Gutierrez, G., et al., *Properties of Lignin, Cellulose, and Hemicelluloses Isolated from Olive Cake and Olive Stones: Binding of Water, Oil, Bile Acids, and Glucose.* J Agric Food Chem, 2014.
201. Kotake-Nara, E. and A. Nagao, *Absorption and metabolism of xanthophylls.* Mar Drugs, 2011. **9**(6): p. 1024-37.
202. Zaripheh, S. and J.W. Erdman, Jr., *Factors that influence the bioavailablity of xanthophylls.* J Nutr, 2002. **132**(3): p. 531S-534S.
203. Goltz, S.R., et al., *Carotenoid bioavailability from raw vegetables and a moderate amount of oil in human subjects is greatest when the majority of daily vegetables are consumed at one meal.* Nutr Res, 2013. **33**(5): p. 358-66.
204. Khachik, F., et al., *Separation and identification of carotenoids and their oxidation products in the extracts of human plasma.* Anal Chem, 1992. **64**(18): p. 2111-22.
205. Khachik, F., P.S. Bernstein, and D.L. Garland, *Identification of lutein and zeaxanthin oxidation products in human and monkey retinas.* Invest Ophthalmol Vis Sci, 1997. **38**(9): p. 1802-11.
206. Lakshminarayana, R., et al., *Possible degradation/biotransformation of lutein in vitro and in vivo: isolation and structural elucidation of lutein metabolites by HPLC and LC-MS (atmospheric pressure chemical ionization).* Free Radical Biology and Medicine, 2008. **45**(7): p. 982-993.
207. Lakshminarayana, R., et al., *Enhanced lutein bioavailability by lyso-phosphatidylcholine in rats.* Mol Cell Biochem, 2006. **281**(1-2): p. 103-10.

Appendices

Appendix 1. Urinary Thiobarbituric Acid Reactive Substances (TBARS) Procedure

Reference: Hye-Sung Lee et al., *Lipids* 27:124-128, 1992

Reagents

5% (w/v) Trichloroacetic acid (TCA) 5 g to 100 mL H_2O.

0.06 M Thiobarbituric acid (TBA). 0.8652 g 2-thiobarbituric acid to 100 mL H_2O.

Store stoppered reagents in cold room. Before use, warm TBA solution in water bath (as the bath heats up) to return all precipitate to solution, then cool to room temperature.

MDA Standard:

10^{-5} M Malondialdehyde (MDA)

Synthesis of MDA

1. Weigh 164.2 mg (1 mmol) malondialdehyde tetramethylacetal into a 100 mL glass stoppered Erlenmeyer flask.
2. Add 100 mL 0.01 N HCl, stopper immediately and wrap the top with Parafilm.
3. Heat in 50 °C water bath for 1hr.
4. Cool to room temperature in a dark place.
5. Dilute with water to 10^{-5} M.

MDA is very sensitive to light, store in the cold room in a flask wrapped in a foil. Approximate shelf life is one month.

Procedure:

1. Label test tubes (medium size, screw-top tubes).

2. Place 3 mL TCA and 1 mL TBA in each tube.

3. Add MDA, H_2O, and sample as shown in a table below.

4. Cap tubes and vortex to mix.

5. Place tubes in rack in 80 °C water bath for 90 min.

6. Remove from bath and cool to room temp.

7. Centrifuge 10 min at 2000 rpm.

8. Read absorbance at 535 nm.

Tube #	MDA Standard		Water	Urine
	µL	µg	µL	µL
1	0	0	1000	
2	50	0.036	950	
3	100	0.072	900	
4	200	0.144	800	
5	400	0.288	600	
6	600	0.432	400	
7	800	0.576	200	
8	1000	0.720	0	
Sample			500	500

Appendix 2. Bile acid extraction and purification

Reference: Locket, PL & Gallaher, DD (1989) Lipids 24, 221-223.

Solutions

Ethanol, 80 %

Chloroform/methanol: Store at room temperature.

Procedure

Extraction

1. Weigh approximately 100 mg of tissue or feces into a screw cap tube (20 X 125 mm or similar size). Record the exact weight.

2. Add 2 mL of absolute ethanol to each tube

3. Sonicate the capped tubes in a bath sonicator for 30 min.

4. Place the tubes in a heating block and reflux at 100°C for 15 min.

4. Centrifuge the tubes at approx. 1,500 X g for 10 min. Transfer the supernatants to screw capped tubes.

5. Suspend the pellets in 2 mL of 80% ethanol, reflux for 15 min in the heating block, and centrifuge as in step 4. Again transfer the supernatants to the screw capped tubes.

6. Suspend the pellet again in 2 mL of chloroform/methanol, reflux for 15 min in the heating block, and centrifuge as in step 4. Transfer the supernatants to the screw capped tubes.

7. Suspend the pellet in 2 mL of chloroform/methanol, centrifuge as in step 4, and transfer the supernatants to the screw capped tubes.

8. Evaporate the pooled supernatants to dryness with nitrogen gas under low heat (<60°C). Store dried samples capped in the freezer until ready for partial purification.

Partial Purification

1. Remove the rack, if present, from inside the solid phase extraction (SPE) column processor. Insert the SPE columns into the column processor and turn on the water aspirator. Adjust the vacuum to about 5-10 mm Hg with the screw located on the vacuum arm closest to the processor.

2. Solvate (activate) the columns by adding 4 mL of MeOH to each column. Follow with 4 mL of distilled water (DW). Do not allow the columns to dry out if possible between solutions. Stop the flow through the columns by releasing the vacuum by opening the wingnut.

3. To tubes containing the dried tissue or fecal extract, add 0.6 mL MeOH and vortex to solubilize the bile acids. Next add 2.4 mL DW to each tube and vortex again.

4. Pour the solution from above through a solvated column and apply vacuum.

5. When the level of solution in the columns drops to the frit, add 3 mL of DW.

6. When the level of DW in the columns drops to the frit, add 3 mL of hexane.

7. When the level of hexane has dropped to the frit for all columns, release the vacuum with the wingnut and carefully remove the cover of the SPE column processor. Insert the rack with labeled tubes in the correct position to collect the column eluents.

8. Add 3 mL MeOH to each column and turn on the vacuum. The flow of MeOH through the column should be about 2-3 mL/min. The MeOH will elute the bile acids off the columns, which will then be collected in the tubes within the processor.

9. After the flow through the columns stops, release the vacuum, remove the tubes, and evaporate the solution to dryness with nitrogen gas at low heat. Cover the tubes with Parafilm and store in the freezer until ready to analyze by HPLC.

Appendix 3. Liver lipid extraction and enzymatic cholesterol assay

1) Weigh out ~1.00 gram of liver tissue and transfer to homogenizing tube.

2) Add 10 mL chloroform:MeOH (2:1).

3) Homogenize for ~15-20 seconds.

4) Filter homogenate through a #1 Whatman filter paper into a glass screw top tube.

5) Rinse homogenizer with 15 mL chloroform:MeOH (2:1) and pick out any tissue trapped on probe.

6) Pour rinse through filter paper into tube.

7) Wash filter paper with an additional 5 mL chloroform:MeOH (2:1).

8) Add 6 mL 0.9% NaCl to filtrate. This represents a volume of 0.2 times the volume of chloroform:MeOH used.

9) Vortex to mix aqueous and non-aqueous phases.

10) Centrifuge for 1 minute at 750 RPM to separate phases. Be careful about placement of tubes in the centrifuge – when rotor spins, long tubes may break as they tilt. Alternatively, let the tubes stand until phases separate.

11) Remove aqueous upper phase with a transfer pipet and discard.

12) Gently layer 3 mL of MeOH:H_2O (1:1). Rinse sides of tubes while adding the MeOH:H_2O.

13) Remove this upper phase with a transfer pipet and discard.

14) Transfer the extracted lipid in chloroform:MeOH (2:1) and transfer rinse to lipid vial.

15) Evaporate Chloroform under N_2. (Gravimetric Step)

16) Reconstitute the fat with a set volume for all samples (10 mL) in Chloroform:MeOH (2:1).

Enzymatic cholesterol assay

Reagents

1) 50 **mM Sodium Phosphate, Dibasic** (MW = 141.96). Make 1 L, pH 6.9 (7.1 g/L).

2) Stock **Phenol Reagent**: 500 mL

Dissolve 3.8 g Phenol crystals in 400 mL Phosphate Buffer. Adjust volume to 500 mL with Phosphate Buffer. Store at 4 °C (up to 1 month).

3) Stock **Mixed Reagent**: 500 mL

Dissolve in 400 mL Phosphate Buffer:

0.203 g 4-aminoantipyrine

1.292 g Sodium Cholate

7.46 g Potassium Chloride

1.0 mL Triton X

Adjust volume to 500 mL with Phosphate Buffer. Store at 4˚C (up to 1 month).

4) **Working Reagent**: (shelf life: 3-4 days)

Reagent	Amt(UI)/30 mL (mL)	Amt(UI)/100 mL (mL)	Amt(UI)/200 mL (mL)
Stock Mixed Reagent	15	50	100
Stock Phenol	15	50	100
Cholesterol Oxidase (UI)	7.5	25	50
Cholesterol Esterase (UI)	7.5	25	50
Peroxidase (UI)	375	1250	2500

Store in ice bath until ready to use.

******NOTE**** For free cholesterol assay, omit Esterase**

A) **Standard Curve:**

Use 1 mg/mL concentration of cholesterol in Chloroform: MeOH (2:1). Use 0, 5, 10, 15, 20, and 25 μL concentrations (depends on cholesterol in sample).

Add 50 μL Triton X-100/Acetone to each tube, Vortex.

Dry under N_2 gas. (Triton Solution = 1.5 g Triton in 10 mL Acetone)

B) **Samples:** Total Cholesterol: 10 μL assayed

Free Cholesterol: 50 μL assayed

Add 50 μL Triton X-100/Acetone.

Dry solvent under N_2 gas.

C) **Assay:** Add 100 μL H_2O to each tube, Vortex.

Add 1 mL Working Reagent/sample, Vortex.

Incubate at 37 ºC for 10 min. Cool to room temperature.

Read absorbance at 500 nm.

Appendix 4. Xanthophyll extraction from dried plant materials and mixed feeds (AOAC official method 970.64)

Chemicals:

1. Extractant: hexane, acetone, absolute alcohol, toluene (10, 7, 6, 7)
2. Methanolic potassium hydroxide, 40%: dissolve 40 g KOH in methanol, cool, and dilute to 100 mL with methanol
3. Sodium sulfate solution 10%: dissolve 10 g anhydrous Na_2SO_4 in 100 mL with H_2O.

Procedure:

1. Sample preparation: 2 g corn meal or DDGS meal; 50 mg marigold meal into 100 mL volumetric flask.
2. Pipet 30 mL extractant into flask, stopper, and swirl 1 min.
3. Hot saponification: pipet 2 mL 40% methanolic KOH into flask, swirl 1min, and place flask in 56°C water bath 20 min.
4. Cool mixture, and let it stand in dark 1 hour, pipet 30 mL hexane into flask, swirl 1 min, dilute to volume with 10% Sodium sulfate solution, and shake vigorously 1 min.
5. Let stand in dark 1 hour before chromatography. Upper phase is about 50 mL.

Appendix 5. Xanthophylls extraction from rat plasma, organs, and urine

Lakshminarayana, R., et al., *Enhanced lutein bioavailability by lyso-phosphatidylcholine in rats.* Mol Cell Biochem, 2006. **281**(1-2): p. 103-10 [207]

Chemicals:

Dichloromethane, methanol (2:1, v:v) containing 2 mM tocopherol

Hexane

Dichloromethane

Ice-cold isotonic saline

10 M KOH

Ice-cold acetone

Plasma samples

1. 0.8 mL of plasma 3 mL of dichloromethane, methanol (2:1, v:v) containing 2 mM tocopherol was added and mixed for 1 min using a vortex mixer
2. 1.5 mL hexane was added, mixed and centrifuged at 1000 g for 5 min
3. The resulting upper hexane/dichloromethane layer was collected.
4. The extraction procedure was repeated twice with 1 mL of dichloromethane and 1.5 mL of hexane.
5. The extract was pooled, evaporated to dryness

Liver, eye, and feed samples

1. These were homogenized separately with nine parts ice-cold isotonic saline using homogenizer and 0.8 mL of homogenate was used for lutein extraction following the procedure as described for the plasma
2. The extracts were saponified separately with 2mL 10 M KOH at 60°C for 45 min. the reaction mixture was vortexes every 15 min during saponification with the addition of 2 mL of ice cold deionized water before lutein extraction as described before

Urine and fecal samples

1. Urine and fecal samples from each rat were aliquot and taken for lutein analysis wither fresh or frozen at -70°C.
2. Urine samples were filtered through No filter paper and fecal matter 2 g was homogenized with ice-cold acetone, diluted suitably and taken for lutein extraction as per the procedure adopted for plasma and tissues.

Appendix 6. SAS code

```
data all_groups   (label = 'All diet groups');


Title2 'Effect of Corn Fermentation Co-Products on lipids and oxidative
stress';
Input rat_number Diet_Group Initial_BW Week_1_BW Week_2_BW KillDay_BW
Week1_FI Week2_FI Week3_FI KillDay_Liver_Weight
KillDay_Epi_fatpad_Weight Cecum_weight
CecumPH Urine_Vol TBARS_ug24hr Fecal_DryWeight
Liver_total_chol_conc_true Liver_total_chol_true fecal_bile_acid


;

Label
rat_number = 'Rat Number'
Diet_Group= 'Diet Group'
Initial_BW = 'Initial Body Weight (g)'
Week_1_BW= 'Week 1 Body Weight (g)'
Week_2_BW=  'Week 2 Body Weight (g)'
KillDay_BW= 'Rat Body Weight At Kill Day (g)'
Week1_FI= ' Food Intake Week 1 (g)'
Week2_FI = 'Food Intake Week 2 (g)'
Week3_FI = 'Food Intake Week 3 (g)'
KillDay_Liver_Weight= 'Rat Liver Weight at Kill day (g)'
KillDay_Epi_fatpad_Weight=    'Kill day Epididymal Fat Pad Weight(g)'
Cecum_weight = ' Cecum Weight At Kill day (g)'
CecumPH     = ' Cecum pH level at Kill day'
Urine_Vol   = 'Urine Volume over 24h Period (ml)'
TBARS_ug24hr = 'TBARS (ug/24hr)'
Fecal_DryWeight = 'Dry Weight of 48h Fecal Collection (g)'
Liver_total_chol_conc_true='Liver total cholesterol conc true (mg/g)'
Liver_total_chol_true='Liver total cholesterol per liver (mg/liver)'
fecal_bile_acid = 'Fecal bile acid (mg/24hr)'
;

output all_groups;


Datalines;
1     1     172   233.5 286.9 336   19.45 23.75 23.75 17.32 3.547
      2.2945      6.65  3.8   4.218 8.6426      11.8948     206.0184
      9.8277
2     1     135.2 197.4 261   315   17.55 20.25 23.35 16.624      1.748
      2.6173      6.36  20.8  6.1984      8.2897      11.5741
      192.4074    7.0551
3     1     175.5 232.3 294.1 337   19.25 21.5  22.4  21.672      2.427
      2.7597      6.74  9.9   7.8408      7.5073      16.3698
      354.7655    6.4524
```

```
4     1     150.5 198.1 249.7 312   17.8  19.65 22.4  17.361
      3.9384      2.4261      6.51  11.9  8.6156      8.2583
      8.5189      147.8973    9.3132
5     1     184.5 237.8 298.4 369   18.95 23.9  23.05 24.59 4.2766
      2.6987      6.52  18.1  14.6972     8.4037      17.0908
      420.2638    4.7307
6     1     171.2 228   270   301.6 21.6  18.35 18.3  14.913
      2.7851      2.2643      6.56  8.3   4.3658      5.8487
      17.054      254.3256    4.8921
7     1     169   223.3 267   304.5 19.68 24.2  22.6  14.69 2.779
      2.4008      6.64  9.8   3.822 6.2148      13.8575     203.5666
      5.3097
8     1     152.6 200   243   273   17.18 20.65 21.25 13.072
      2.7025      1.6909      6.75  12.5  4.5   5.6574      11.0013
      143.8093    4.8277
9     1     170   220.2 264.2 299   20.16 20.15 17.65 13.31 2.6023
      3.1632      6.26  5.1   3.8046      5.8833      14.0759
      187.3501    6.7617
10    1     180   237.9 293   337   22.5  21.7  20.2  15.29 4.9552
      2.9366      6.4   12.4  7.7624      6.2111      10.6652
      163.0709    7.0495
11    2     155.2 209.9 271.5 324   21.3  23.7  25.6  15.022      2.573
      3.6274      6.81  18.9  9.261 7.7438      3.3613      50.4941
      24.0867
12    2     181   239   295.6 335   23.85 23.95 24.3  17.457      2.781
      3.0729      6.42  17.6  8.8704      7.5012      8.6083
      150.2755    21.1361
13    2     162.4 216   276.3 339   20.15 23.1  27.15 17.91 4.261 3.178
      6.89  15.8  10.8388     7.5386      7.1905      128.7827
      23.4623
14    2     155.2 202.2 253.8 319   19.6  23.2  24.7  13.647      3.755
      2.9455      6.41  25.1  8.3332      7.0734      5.0847
      69.3915     17.6705
15    2     160.7 204.6 237.4 275   17.55 17.55 20.95 12.889
      2.4526      3.0336      6.4   1.9   2.0596      5.0646
      5.7007      73.4765     17.2892
16    2     167.2 226   273.6 311   18.55 22.45 22.5  15.568      4.166
      4.376 6.38  10.6  6.6144      5.9843      8.8294      137.4556
      24.7868
17    2     157.7 200   235.4 268   16.35 20.05 19.63 11.284
      2.1296      2.5608      6.49  4.7   3.1208      5.8801
      9.3142      105.1017    23.5001
18    2     177   232   273   309   22.25 23.7  26    14.715
      2.9433      3.4021      6.44  8     5.088 5.7684      10.8358
      159.4482    22.9034
19    2     190.5 255.2 304.6 357   24.15 20.45 26.75 16.79 3.7785
      3.29  6.62  1.9   0.8892      7.1814      4.8818      81.9649
      32.85
20    2     187.7 243.1 285.1 344   25.25 26.15 20.95 15.89 3.6192
      3.5057      6.65  22.2  3.108 8.2089      7.8449      124.6555
      32.1769
21    3     160.5 213.2 279.5 305   20.2  25.2  24.3  14.402      3.65
      1.7089      6.49  8.4   6.636 8.3488      12.1428     174.8805
      39.3389
```

```
22    3     178.8 232   288   342   21    22.9  28    19.165      4.099
      3.1074      6.62  33.9  10.509      6.7813      6.0189
      115.3525    34.3836
23    3     160   206   255.2 286   19.4  19.3  25.05 18.447      2.086
      2.7576      6.47  3.8   3.4352      6.1354      12.7819
      235.7873    29.4122
24    3     160   220.3 280   344   21.8  23.45 25.15 21.298      3.653
      2.7382      6.76  9.2   6.5504      6.5617      13.4122
      285.6528    26.2007
25    3     157.6 202.6 267.8 333   20.15 23.55 26.95 16.912
      2.5825      2.7282      6.43  6.6   4.356 7.1069      5.4501
      92.1725     33.6072
26    3     175.5 262.5 266.7 303.3 19.5  17.65 20    15.05 3.6807
      2.8181      6.67  26.9  2.4748      7.0094      6.8113
      102.5105    35.0551
27    3     173.2 223.8 266.8 305   16.95 19.75 23    14.139
      2.5517      2.256 6.65  9.9   6.435 6.7372      9.0232
      127.5787    30.0285
28    3     160.2 222.2 265   294.2 21.6  20.25 22.75 15.08 3.9036
      2.6341      6.55  7.6   6.0952      5.9482      13.875
      209.2354    33.2982
29    3     170.7 216.6 250   289   20.4  22.25 23.75 12.92 2.612
      2.3051      6.34  7.2   7.128 5.4685      8.5374      110.3036
      19.6834
30    3     153.2 205.2 253.4 298   19.05 19.6  17.35 13.71 3.631
      2.6328      6.5   4.5   4.383 4.9887      10.2943     141.1342
      19.839
31    4     151.2 199.1 247.6 287   19.2  20.7  21.85 15.949      2.084
      .     6.47  13.2  6.1512      5.3606      6.2776      100.1219
      16.4439
32    4     167.2 223.2 288.7 355   18.85 26.7  26.9  20.719      3.396
      4.5282      6.29  24.8  4.3648      7.1998      6.0856
      126.0878    20.1484
33    4     165.6 218   265.7 304   18.6  18.45 21    15.045      2.565
      3.5514      6.76  8.2   5.4776      5.2392      4.0845
      61.451      16.5192
34    4     166.4 218.7 267.9 320   19.9  20.25 25.7  18.358
      2.7345      3.7853      6.61  9.8   5.1352      6.7196
      4.9804      91.4301     19.1537
35    4     169.6 221.3 278   323   19.8  22.7  22.3  16.965      3.19
      2.8611      6.82  8.6   5.9168      6.1466      7.9453
      134.7921    17.0482
36    4     177.9 245.5 294.6 339   21.85 25.25 21.15 17.2355
      3.4246      4.8746      6.46  5     5.28  6.4707      9.8354
      169.5182    19.8781
37    4     149.7 182.2 212.8 233   13.95 15.35 17.05 10.35 2.145
      3.0268      6.44  15.2  7.144 5.0797      4.6729      48.3645
      17.1353
38    4     180.6 230   269.2 283   20.2  21.75 23    8.82  2.3145
      1.364 7.22  19.8  4.7916      5.1623      12.8482     113.3209
      16.1542
39    4     156.3 212   242.8 284   17.8  19.05 20.8  14.73 2.8533
      3.8358      6.01  4.3   6.0028      6.3534      6.6647
      98.1707     16.3819
```

```
40    4     174.1 229.4 273.6 311   21.9  22.8  22.5  14.85 3.663
      3.7085      6.67  7.5   5.85  6.434 7.3145      108.6207
      14.5146

;

proc sort data = all_groups; by Diet_Group;

proc format;
      value dietfmt
            1='Control Diet'
            2='DDGS Diet'
            3='Bran fraction Diet'
            4='Soluble fraction Diet'
            ;

proc print data= all_groups; by Diet_Group; format Diet_Group dietfmt.;
proc means data=all_groups n mean stderr min max; by Diet_Group; format
Diet_Group dietfmt.;
var Initial_BW--fecal_bile_acid;
run;
proc glm data= all_groups;
title 'One Way Analysis of Variance in all rats';
format Diet_Group dietfmt.;
class Diet_Group;
model Initial_BW--fecal_bile_acid=Diet_Group;
Means Diet_Group / Duncan;
run;
proc corr data=all_groups;
title 'Correlation of Fecal Bile Acids and Liver Cholesterol in all
rats';
format Diet_Group dietfmt.;
var Liver_total_chol_conc_true Liver_total_chol_true;
with fecal_bile_acid;
run;

quit;
```

Appendix 7. SAS code for Neutral Sterols Analysis

```
data all_groups   (label = 'All diet groups')
;
Title1 'bioavailability of DDGS';
Input Rat_No Diet_Group Feces_weight_mg coprostenal_conc
cholesterol_conc cholestanol_conc oneday_feces_weight_g
coprostenal_in_24hours_feces cholestrol_in_24hours_feces
cholestnol_in_24hours_feces coprostenal_feces_umol
cholesterol_feces_umol cholestnol_feces_umol
total_neutral_sterol_umol_day
;

Label
Rat_No = 'Rat Number'
Diet_Group= 'Diet Group'
Feces_weight_mg='feces weight (mg)'
coprostenal_conc='coprostenal conc (ug/mg)'
cholesterol_conc=' cholesterol conc (ug/mg)'
cholestanol_conc='cholestanol conc (ug/mg)'
oneday_feces_weight_g= '24h feces weight (g)'
coprostenal_in_24hours_feces='coprostenal in 24hours feces (mg)'
cholestrol_in_24hours_feces='cholestrol in 24hours feces (mg)'
cholestnol_in_24hours_feces='cholestnol in 24hours feces (mg)'
coprostenal_feces_umol='coprostenal in 24hours feces (umol)'
cholesterol_feces_umol='cholestrol in 24hours feces (umol)'
cholestnol_feces_umol=' cholestnol in 24hours feces (umol)'
total_neutral_sterol_umol_day='total neutral sterol (umol/day)'
;



Datalines;
1     1     53.820      1.175 0.379 0.198 2.881 3.39  1.09  0.57  8.71
      2.83  1.47  13.01
2     1     53.380      1.677 3.145 0.348 2.763 4.64  8.69  0.96  11.93
      22.47 2.48  36.88
3     1     51.430      1.803 3.301 0.322 2.502 4.51  8.26  0.81  11.61
      21.37 2.08  35.05
4     1     50.410      1.105 4.113 0.142 2.753 3.04  11.32 0.39  7.83
      29.28 1.01  38.11
5     1     49.870      1.557 3.378 0.275 2.801 4.36  9.46  0.77  11.22
      24.47 1.99  37.69
6     1     50.080      1.617 2.883 0.315 1.950 3.15  5.62  0.61  8.11
      14.54 1.58  24.23
7     1     53.380      1.563 2.997 0.284 2.072 3.24  6.21  0.59  8.33
      16.06 1.52  25.91
```

```
8     1     48.910      1.150 2.608 0.218 1.886 2.17  4.92  0.41  5.58
      12.72 1.06  19.36
9     1     47.870      1.161 2.112 0.259 1.961 2.28  4.14  0.51  5.86
      10.71 1.31  17.88
10    1     48.750      1.050 2.896 0.187 2.070 2.17  6.00  0.39  5.59
      15.51 1.00  22.10
11    2     51.340      3.862 3.322 0.719 2.581 9.97  8.58  1.86  25.65
      22.18 4.78  52.61
12    2     51.520      1.879 2.637 0.561 2.500 4.70  6.59  1.40  12.09
      17.05 3.62  32.76
13    2     52.770      2.065 2.200 0.521 2.513 5.19  5.53  1.31  13.35
      14.30 3.38  31.02
14    2     51.000      1.917 2.526 0.420 2.358 4.52  5.96  0.99  11.63
      15.40 2.55  29.59
15    2     52.380      2.638 3.014 0.493 1.688 4.45  5.09  0.83  11.46
      13.16 2.15  26.77
16    2     50.850      2.943 2.090 0.556 1.995 5.87  4.17  1.11  15.10
      10.78 2.86  28.74
17    2     50.850      0.887 1.120 0.164 1.960 1.74  2.19  0.32  4.48
      5.68  0.83  10.98
18    2     54.640      2.576 2.622 0.403 1.923 4.95  5.04  0.78  12.74
      13.04 2.00  27.78
19    2     49.630      2.050 1.812 0.379 2.394 4.91  4.34  0.91  12.63
      11.22 2.34  26.19
20    2     54.630      2.574 2.083 0.520 2.736 7.04  5.70  1.42  18.12
      14.74 3.67  36.53
21    3     49.470      2.858 1.832 0.565 2.783 7.95  5.10  1.57  20.46
      13.19 4.05  37.70
22    3     52.070      2.827 2.877 0.624 2.260 6.39  6.50  1.41  16.44
      16.82 3.64  36.90
23    3     50.310      3.305 2.362 0.578 2.045 6.76  4.83  1.18  17.39
      12.49 3.05  32.94
24    3     53.420      2.883 2.564 0.708 2.187 6.30  5.61  1.55  16.22
      14.50 3.99  34.72
25    3     51.300      2.650 2.613 0.516 2.369 6.28  6.19  1.22  16.15
      16.01 3.15  35.31
26    3     54.070      1.191 0.928 0.250 2.336 2.78  2.17  0.58  7.16
      5.61  1.51  14.27
27    3     47.980      1.143 1.344 0.187 2.246 2.57  3.02  0.42  6.61
      7.81  1.08  15.50
28    3     49.750      1.719 1.533 0.378 1.983 3.41  3.04  0.75  8.77
      7.86  1.93  18.56
29    3     53.600      1.515 1.981 0.291 1.823 2.76  3.61  0.53  7.11
      9.34  1.36  17.81
30    3     49.390      1.561 1.615 0.338 1.663 2.60  2.69  0.56  6.68
      6.95  1.45  15.07
31    4     52.070      4.836 2.661 0.945 1.787 8.64  4.76  1.69  22.23
      12.30 4.35  38.88
32    4     54.220      4.578 3.195 0.910 2.400 10.99 7.67  2.18  28.27
      19.83 5.63  53.73
33    4     51.230      3.146 3.054 0.631 1.746 5.49  5.33  1.10  14.14
      13.80 2.84  30.77
34    4     53.120      2.633 1.431 0.452 2.240 5.90  3.20  1.01  15.17
      8.29  2.61  26.07
```

```
35    4     54.780      4.207 2.162 0.761 2.049 8.62  4.43  1.56  22.18
      11.46 4.02  37.66
36    4     47.500      2.105 0.810 0.321 2.157 4.54  1.75  0.69  11.68
      4.52  1.78  17.98
37    4     48.910      0.662 0.251 0.088 1.693 1.12  0.42  0.15  2.89
      1.10  0.38  4.37
38    4     48.450      4.700 1.974 0.706 1.721 8.09  3.40  1.22  20.81
      8.79  3.13  32.73
39    4     51.830      0.765 0.257 0.098 2.118 1.62  0.54  0.21  4.17
      1.41  0.54  6.12
40    4     54.000      1.187 1.708 0.201 2.145 2.55  3.66  0.43  6.55
      9.47  1.11  17.14


;

proc sort data = all_groups; by Diet_Group;

proc format;
      value dietfmt
            1='control diet'
            2='DDGS diet'
            3='bran fraction diet'
            4='soluble fraction diet'
            ;


proc print data= all_groups; by Diet_Group; format Diet_Group dietfmt.;

proc means data=all_groups n mean stderr min max; by Diet_Group; format
Diet_Group dietfmt.;

proc glm data= all_groups;
title 'One Way Analysis of Variance in all rats';
format Diet_Group dietfmt.;
class Diet_Group;
model Feces_weight_mg--total_neutral_sterol_umol_day =Diet_Group;;
Means Diet_Group / Duncan;
LSMeans Diet_Group / STDERR pdiff;
run;
quit;
```